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EXTRA-EMBRYONIC ENDODERM OF THE AVIAN EMBRYO: DIFFERENTIATION POTENTIAL AND ANTIGEN EXPRESSION

by

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ABSTRACT

The lack of studies of the developing avian yolk sac led to an examination of the differentiation potential and antigenic expression of the extra-embryonic endoderm of the chick embryo.

Extra-embryonic endoderm from stage 5 and stage 22 chicken embryos (Hamburger and Hamilton, 1951) was grafted to the chorioallantois of embryonated eggs, and incubated for 4 or 8 days. In contrast to control tissues from the area pellucida of stage 5 embryos, the extra-embryonic tissues failed to penetrate the host chorioallantoic membrane. Yolk-sac-like cells were the sole differentiation product observed, suggesting that extra-embryonic endoderm is committed to form yolk sac at a very early developmental stage. The failure of the yolk sac endoderm to penetrate the chorioallantois may be related to its precocious development.

Specific antisera were raised against stage 5 and stage 22 extra-embryonic endodermal cells, in an attempt to characterize tissue-specific antigens. Absorbtion analyses of the sera indicated the presence of several antigens common to the yolk, egg white protein, extra-embryonic endodermal cells, embryonic chicken serum, and adult chicken serum. Antisera to stage 5 and stage 22 extra-embryonic endoderm detected at least one saline-soluble antigen that is specific for extra-embryonic endoderm. Immunofluorescence studies indicated the presence of antigens common to the cytoplasm of extra-embryonic cells, adult bone marrow, and adult heterophils. The possibility that the antisera detect a developmental antigen is discussed.

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INTRODUCTION

Four major extra-embryonic membranes are formed during avian development: the chorion, the yolk sac, the amnion and the allantois (Patten, 1964; Balinsky, 1975; Rugh, 1964) (see Figure 1). They are derived from the extra-embryonic region of the blastoderm, and function as highly specialized organs during the embryonic period. The chorion actively transports calcium (Terepka, 1969) and when fused to the allantois, serves as the embryonic respiratory organ. The yolk sac absorbs and transports nutrients from the yolk to the embryo and induces blood formation. The allantois is the receptacle for embryonic wastes and the amnion protects against mechanical shock and dessication (Romanoff, 1960). At hatching, all of the extra-embryonic membranes, with the exception of the yolk sac, degenerate and are discarded. The yolk sac is retained within the chick as a duodenal caecum (Kar, 1947; Romanoff, 1960).

The yolk sac is the first extra-embryonic membrane to develop, originating at the time of gastrulation. The bilaminar blastodisc, composed of epiblast and hypoblast, exhibits two morphologically distinct regions. The outer area opaca forms the extra-embryonic region of the developing embryo; the lighter area pellucida, located centrally, forms the embryonic region. During gastrulation, the invagination of endoblast (definitive endoderm) (Nicolet, 1967; Rosenquist, 1972; Vakaet, 1962; Wakely and England, 1978) results in the displacement of the hypoblast laterally and anteriorly. This outward movement of the hypoblast occurs in concert with the radial growth of the extra-embryonic ectoderm. The result is a

steady expansion of the extra-embryonic region as the two cell layers move over the yolk surface. By the end of the fourth day of incubation, the yolk is totally surrounded by extra-embryonic cells.

A third cell population, the mesoderm, is necessary to complete the development of the yolk sac. Located between the embryonic ectoderm and the embryonic endoderm, the mesoderm migrates laterally, splitting into a dorsal, somatic and a ventral, splanchnic cell sheet. Both cell sheets continue to migrate into the extra-embryonic region. The somatic mesoderm forms the bulk of the chorion, while the splanchnic sheet interacts with the extra-embryonic endoderm. The result of this interaction is the formation of blood islands within the extra-embryonic splanchnic mesoderm, and the formation of yolk sac villi by the extra-embryonic endoderm.

Examination of the yolk sac during the third or fourth day of incubation reveals two morphologically distinct regions: a proximal region containing yolk sac villi and blood islands, and an avascular, distal region composed of irregularly arranged cells (see Figure 2). The avascular region is termed the area vitellina; the vascular region is the area vasculosa. Vascularization of the entire yolk sac is achieved by the end of the fifth day of incubation, coinciding with the completion of mesodermal migration throughout the extra-embryonic region.

There are few detailed studies of yolk sac development. Romanoff (1960) noted that '... physiological activity of the yolk sac endoderm is correlated with morphology. The ability to secrete enzymes evolves

gradually with the organization of the cells into an epithelium.' Lambson (1970) reported that yolk absorbtion occurred in both organized and unorganized extra-embryonic endoderm. Grodzinski (1930) observed phagocytosis of India ink granules in monolayer cultures of extra-embryonic endoderm from the area vitellina. Thus, yolk sac endoderm, whether organized into an epithelium or not, is capable of absorbing yolk materials. As such, it is functionally differentiated.

Histochemical studies of yolk sac development were carried out by Juurlink and Gibson (1973). Prior to the third day of incubation, they failed to detect any tissue-specific enzyme activity or cell constituent within the yolk sac cells. At three days of incubation, both alkaline and acid phosphatases were detectable; at five days, the activity of these two enzymes had increased, and ribonuclease activity was observed. Unfortunately, Juurlink and Gibson restricted their study to the area vasculosa and do not refer to the area vitellina or area opaca of earlier embryos.

Bennett (1973) detected an enzyme that is specific for yolk sac endoderm, cysteine lyase. The enzyme was first observed in the proximal extra-embryonic cells of stage 5 embryos ('advanced primitive streak', Bennett, 1973). In later stages of development, the enzyme is located in progressively distal regions of the extra-embryonic endoderm. The presence of the enzyme is closely linked with the migration of the extra-embryonic splanchnic mesoderm. Activity of the enzyme is highest in the proximal regions of the area vasculosa; there is minimal activity in the area vitellina. Sanders and Dickau (1978) attempted unsuccessfully to

find cysteine lyase in the area opaca of unincubated embryos (stage XIII, Eyal-Giladi and Kochav). They also tested incubated eggs to stage 3 (Hamburger and Hamilton, 1951) with similar results. Given the association between cysteine lyase, and the presence of splanchnic mesoderm (Bennett, 1973), these results are not surprising. There is no extra-embryonic endoderm in the chick embryo during the developmental period studied by Sanders and Dickau (England and Wakely, 1977).

Morphological studies of yolk sac development were made by Virchow (1891) and Bellairs (1963). Both authors noted that the early morphogenesis of the yolk sac is simply a matter of arranging the newly formed extra-embryonic endodermal cells into an epithelium. The growth of the yolk sac over the yolk surface is centrifugal, accomplished by rapid division of the distal endodermal cells. Romanoff (1960) described this peripheral region as a zone of free nuclear division. Bellairs (1963) disputed this claim, and observed that there were no free nuclei in the region. Instead, she described the region as one exhibiting rapid cellular proliferation, Ruth (1978) referred to the region as 'blastema-like'.

Bennett (1973) measured the mitotic index of cells of the extra-embryonic endoderm using stage 10 chicken embryos. She reported that seventy-six per cent of the peripheral cells exhibited mitosis. The number of cells in mitosis decreased dramatically in the proximity of the embryo. Proximal cells of the extra-embryonic endoderm had a mitotic index of four per cent, while cells in the medial region of the yolk sac had a mitotic index of thirty per cent. Bennett noted

a relationship between the low mitotic index of cells containing cysteine lyase and the presence of mesoderm in the extra-embryonic region. She suggested that the decrease in mitotic index was related to the stage of tissue differentiation.

The work of Bennett (ibid) suggested that the yolk sac endoderm may be determined, or committed in its developmental pathway, at a very early stage in development. In the first portion of this thesis, the differentiation potential of the extra-embryonic endoderm is examined using chorioallantoic grafting as the assay method. If yolk sac endoderm is committed in its developmental pathway, it should form only yolk sac tissue. Presence of other endodermal tissues, such as duodenum or other gut derivatives would suggest that the yolk sac is capable of forming tissues other than yolk sac, and that the endoderm is not completely committed.

The second question that is studied in this thesis also concerns the differentiation of the yolk sac. The inability of previous investigators to identify a specific marker of extra-embryonic endodermal differentiation with histochemical and morphological techniques led to an examination of alternate methods of tracing cell differentiation. The alternate method chosen was the use of specific antiserum.

Wolk and Eyal-Giladi (1977) prepared antisera against hypoblast and epiblast of stage XIII chicken embryos (Eyal-Giladi and Kochav, 1976). They detected an antigen specific for the hypoblast cells. The presence of this antigen was noted in embryos from stage X, XI, XII, and XIII (ibid). Later embryos were also tested with the antisera.

The authors concluded that the antisera could be used to follow morphogenetic movements.

Two possible pathways of yolk sac differentiation are suggested by the literature. The first possibility is that the yolk sac endoderm is fully differentiated shortly after it is created. Differentiated cells would not be expected to possess a series of cellular antigens that would change during the incubation period. Antigens found in these cells would be 'persistent' or present throughout development. The second possibility is that the yolk sac cells undergo a series of developmental changes prior to becoming fully differentiated. Hopefully, these changes would involve the antigenic composition of the yolk sac endoderm. Any alterations might be observed through the use of specific antisera, raised against the extra-embryonic endoderm.

CHAPTER ONE

DIFFERENTIATION OF EXTRA-EMBRYONIC ENDODERM GRAFTED TO THE CHORIOALLANTOIS

Introduction

Embryonic endoderm can differentiate into several different cell types including oesophagus, gizzard and intestine. It can also form yolk sac (Sumiya, 1976). Conversely, the extra-embryonic endoderm is apparently restricted to the formation of absorbtive yolk sac villi, which function in phagocytosis and the transport of yolk proteins (Bellairs, 1963). There are no published reports on the ability or inability of extra-embryonic endoderm to form embryonic structures. In the absence of such reports, we have examined the differentiation potential of extra-embryonic endoderm that has been grafted to the chicken chorioallantois.

The use of chorioallantoic grafting as a means of determining tissue competence originated with the studies of Danchakoff (1920). She noted that inoculation of the chorioallantois with embryonic tissues led to vascularization of the graft by the host embryo. Furthermore, grafted tissues differentiated to form histologically identifiable tissues and organs.

Chorioallantoic grafting proved to be an excellent technique for studying differentiation because it was readily repeatable, permitted manipulation of tissues on the membrane, and the donor tissues could be readily observed during the incubation period. Numerous investigators - Hoadley (1926a; 1926b; 1926c), Willier (1930), Murray and Selby (1930), Dalton (1935), Waddington (1934), Rudnick and Rawles (1937), Rudnick (1932; 1935; 1944), and DeLanney and Ebert (1962) - have used this technique to study the differentiation potential of virtually all embryonic cell and tissue types during development.

More recently, investigators have used chorioallantoic grafting to study the graft-versus-host reaction (Longenecker *et al.*, 1970) and angiogenesis (Folkman, 1975). In this section of the thesis, the differentiation of extra-embryonic endoderm from stage 5 and stage 22 chicken embryos (Hamburger and Hamilton, 1951) was examined using the chorioallantoic grafting method.

Avian embryos are identified on the basis of morphological criteria set forth by Hamburger and Hamilton in 1951. Stage 5 embryos are characterized by a 'pear-shaped' area pellucida, the primitive pit, and by the presence of the primitive groove and Hensen's node (Hamilton, 1952). The notochord is present as a thin strip of condensed chordomesoderm that extends anteriorly from Hensen's node. This stage of development is attained after eighteen to twenty-one hours incubation at 38°C (Hamilton, 1951). Stage 22 embryos have limb buds, distinct eye pigmentation and a well-developed allantois. Three and one-half to four days of incubation are required to reach stage 22 (Hamilton, 1952).

It is reasonable to suggest that the earlier stage 5 extra-embryonic endoderm may exhibit a greater differentiation potential than the older, stage 22 endodermal tissues. If this is so, it might be expected to show greater variability in its differentiation products. A priori, there is no reason to assume that the tissues are the same.

Materials and Methods

Eggs of outbred White Leghorn chickens, purchased from the University of Alberta farm, were used throughout the experiments.

Donor embryos were incubated at 38°C for seventeen to nineteen hours (stage 5, Hamburger and Hamilton 1951) or ninety-six hours (stage 22, ibid) in a Jamesway forced air incubator (James Manufacturing Company, Wisconsin). Stage 5 embryos were removed from the yolk and placed in ice-cold Pannett and Compton saline (Pannett and Compton, 1924; see Appendix I). The solution was modified by substituting 15 mM HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma; pH 7.5) for the original phosphate salts. After one hour in the cold saline, the vitelline membranes were removed from the embryos. Adherent yolk was removed by repeated washing in cold saline.

The anterior opaque areas (a. opacae) were trimmed from the embryos using fine iridectomy scissors and were stored on ice while the host embryos were prepared. Immediately before transfer to the host embryos, a. opaca tissues were cut into four to six pieces of similar size (6.0 - 12 mm^2) and transferred to 37°C saline in order to reduce potential cold shock to the host.

Stage 22 embryos were placed in bowls of warm saline and the yolk sac was carefully removed. After washing in ice-cold saline, the endodermal cells of the vitelline areas were removed from the extra-embryonic ectoderm using steel dissection needles. These endodermal sheets were washed in saline to remove yolk and then stored on ice. Prior to grafting, the endodermal sheets were cut into sections measuring 2 - 3 mm square, and transferred to 37°C saline.

Eggs containing host embryos were incubated for ten days at 38°C. The eggs were then removed from the incubator, placed in a laminar flow hood, and prepared as described by Coulombre (1967). Host viability was determined by candling. Viable eggs were swabbed with an alcoholic iodine solution (1 gm KI; 100 ml 70% ethanol). The blunt end of the egg was punctured using an 18 gauge needle; a second puncture was made through the shell near the mid-point of the longitudinal axis of the egg. Suction was applied to the blunt end of the egg, 'dropping' the chorioallantoic membrane from the shell membrane. Eggs were discarded if their membranes failed to drop, or if air bubbles were present in the extra-embryonic region.

With the egg held horizontally, a 5 mm x 5 mm window was outlined on the egg shell. A dental drill with abrasive disc was used to cut the window. Shell sections were removed with forceps and the shell membrane was carefully peeled away. The window was covered with masking tape and the eggs were returned to the incubator in a horizontal position.

After approximately one hour, the host embryos were taken from the incubator and the tape removed. The tissue segments previously prepared were drawn into sterile Pasteur pipettes and dropped onto the surface of the chorioallantoic membrane. One tissue fragment was added to each host embryo. The tape was replaced, and the window sealed with Paraplast (Sherwood Medical Industries, Missouri). Host embryos were incubated for either four or eight days, at which time their chorioallantoic membranes were harvested. Control embryos were incubated concurrently with those embryos receiving experimental tissues. Negative controls consisted of unopened eggs, fenestrated

eggs and fenestrated eggs inoculated with saline. Positive controls were inoculated with tissues from the nodal region of a stage 5 embryo.

Following harvesting, control and experimental membranes were rinsed in warm saline, fixed in buffered formalin (Humason, 1972) or Bouin's fixative (ibid), and dehydrated in a standard series of alcohols. Tissues were cleared in trichloroethylene or benzene and embedded in Paraplast. Sections were cut at 7 to 8 microns. Ehrlich's hematoxylin and eosin, and Mallory-Heidenhain stains were used most frequently (see Appendix II). Schedules for these stains appear in Humason (1972). Photomicrographs were taken using a Leica camera mounted on a Wild M-20 microscope. Kodak Panatomic-X film (ASA 32) was used throughout this portion of the study; developing and printing were done commercially.

Autoradiography

Sheets of a. opacae from the anterior portion of stage 5 embryos were rinsed in ice-cold Pannett and Compton saline and placed in Petri plates (Falcon Plastics #1008) containing 1 mL of Medium L-15 (GIBCO) with fifty microliters of tritiated thymidine [50 microcuries methyl (^{3}H-thymidine), New England Nuclear; specific activity = 6.7 Ci/mmol.] Tissue sections were incubated for one hour at 38°C in a Hotpack incubator. The medium was replaced with fresh L-15 medium containing 29 mM thymidine (Sigma) and the incubation was continued for another hour.

Labelled tissues were grafted to hosts as previously described. Untreated or sham-labelled extra-embryonic stage 5 endoderm was used as control tissue. Sham-labelled tissues were incubated in

L-15 medium containing 29 mM thymidine for one hour at 38°C. At this point, the medium was replaced with fresh 1-15 medium containing 29 mM thymidine and the incubation was continued for one more hour. Control tissues were grafted as described above.

Labelled grafts and control membranes were fixed in formaline, dehydrated and embedded in Paraplast. Sections were cut at 5 microns. Slides were dried for four hours on a warming tray, then stored at 4°C for up to two weeks. The sections were then rehydrated through an alcohol series, ending in distilled water. Slides were dip-coated in Kodak NTB-3 liquid emulsion, and allowed to air dry for one hour in complete darkness. Dry slides were stored in Bakelite slide boxes which were sealed with tape, wrapped in foil, and exposed at 5°C for twenty-three days. Slides were developed in Kodak D-11 developer (Hornsby, 1975; Prescott, 1964) for three minutes at 4°C, rinsed with running tap water for ten seconds and fixed for ten minutes in 4°C Kodak Rapidfix. A final rinse for fifteen minutes in cold running tap water completed the development process and ensured that the grains would not fade. Slides were stained with Ehrlich's hematoxylin and eosin, and mounted in DPX mountant.

Results

A summary of graft survival is presented in Table 1. In general, grafts survived extremely well, averaging 87 per cent. Grafts were considered to have survived if the graft was vascularized by at least one host vessel, and if there was no evidence of infection either in the host or the graft.

Figure 4 shows the pattern of vascularization for a typical extra-embryonic graft on the chorioallantois. One or two major vessels usually penetrate the graft region, with smaller vascular branches appearing in the surrounding graft tissue. These smaller branches arise from the major vessels, but do not penetrate the graft. Instead, they occupy the circumferential and dorsal aspects of the graft. It is of interest to note that vessels associated with the graft do not form vascular loops (Ausprunk and Knighton, 1975), but approach the graft directly.

Chorioallantoic membranes from unopened hosts were used as normal controls. The chorionic epithelium of these membranes is composed of stratified cuboidal cells containing ellipsoid nuclei in an eosinophilic, agranular cytoplasm. A vascular sinus is seen between the upper and lower layers of the epithelium (Leeson and Leeson, 1963; Ganote *et al.*, 1964; Sethi and Brooks, 1971; D'Aunoy and Evans, 1937).

Beneath the chorionic epithelium lies the mid-region of the chorioallantois, composed of mesoderm from both the chorion and the allantois. The bulk of this region is acellular, staining pale blue with hematoxylin, and purple with the Mallory-Heidenhain stain. Cellular components are typically fibroblastic, possessing the stellate cell bodies and fine processes that characterize these cells. Vessels

TABLE 1

SURVIVAL OF EXTRA-EMBRYONIC ENDODERM GRAFTED TO THE CHORIOALLANTOIS

	Donor Tissue	Viability for each Graft Period	
		4 days	8 days
Graft	stage 5 endoderm, a. opaca	23/27 (3)	22/25 (4)
Experiments	stage 22 endoderm, a. vitellina	11/13 (3)	14/16 (4)
Control	non-fenestrated	6/6 (1)	
Experiments	fenestrated	4/4 (1)	
	fenestrated + saline	5/6 (1)	
	fenestrated + saline + nodal tissue	4/4 (1)	
Autoradiography	stage 5 endoderm, a. opaca + ^{3}H-thymidine	4/6 (2)	
	stage 5 endoderm, a. opaca + thymidine	5/6 (2)	
	stage 5 endoderm, a. opaca	3/6 (2)	

$$\text{Viability} = \frac{\text{\# grafts recovered}}{\text{\# grafts attempted}}$$

(n) = number of experiments.

are most commonly found in the upper portions of the mesodermal region, near the interface of the mesoderm and the chorionic epithelium.

A simple cuboidal epithelium forms the ventral surface of the chorioallantois. The cells of this region are larger than their counterparts in the chorionic epithelium, and are easily identified by the presence of dark cytoplasmic granules near their apical ends. In general, these cells are slightly more basophilic than the cells of the chorionic epithelium.

Preliminary experiments showed that fenestration of the host's shell led to keratinization of the chorioallantois. A window of 5 mm x 5 mm was found to be the smallest aperature that combined ease of grafting with minimal keratinization. Maximal keratinization occurred immediately below the shell window; the intensity of the reaction decreased further away from the shell opening.

Inoculation of host embryos with warm saline resulted in several gross modification to the chorioallantois. These included the formation of two types of cellular structures within the membrane, hyperplasia of the chorionic epithelium, and a metaplastic reaction within the mesodermal region of the membrane.

Chorionic hyperplasia is shown in Figure 6. Both pappilliform processes and squamous stratification were commonly seen in inoculated hosts. These reactions were accompanied by a massive swelling of the chorioallantois in the region immediately below the graft. Figure 7 shows the changes in the mesodermal region.

Two forms of epithelial inclusions were found in the membranes of saline-treated hosts. The first of these, termed epithelial

cell nests, consisted of a mass of chorionic epithelial cells within the mesodermal region of the membrane. A poorly defined fibroblastic capsule was occasionally seen surrounding these cell nests. The second group of inclusions were epithelial pearls (Moscona, 1959a). These structures were similar to the cell nests in size, but differ in that they were only found at the interface of the chorionic epithelium and the graft, or at the mesoderm-chorionic epithelium interface. In addition, these structures were keratinized and highly eosinophilic. Figure 8 shows examples of epithelial cell nests and epithelial pearls.

Control embryos that received embryonic tissues were vascularized by a series of looping vessels. These vessels penetrate the graft directly. Epithelial cell nests, epithelial pearls, mesodermal metaplasia, epithelial hyperplasia and keratinization were observed in host membranes (see Table 2). Figure 9 shows differentiated control tissues within the chorioallantois. The tissues most commonly observed were optic cup, retina, and neural tube. Notochord was occasionally seen.

Membranes of host embryos inoculated with either stage 5 or stage 22 extra-embryonic endoderm exhibit keratinization, mesodermal swelling, chorionic hyperplasia and mesodermal metaplasia. Epithelial cell nests and epithelial pearls were occassionally seen (see Tables 3 and 4).

In contrast to control tissues, extra-embryonic tissue did not penetrate the host chorioallantois. The graft lay upon the chorionic epithelium; its center was necrotic. PAS-positive yolk

TABLE 2

FREQUENCY OF MODIFICATIONS TO THE CHORIOALLANTOIS OF CONTROL EMBRYOS

Structure	Non-Fenestrated (6)	Fenestrated (4)	Fenestrated + Saline (5)	Fenestrated + Saline + Nodal tissue (4)
keratinization	0	4	5	4
ectodermal hyperplasia	0	0	5	4
mesodermal swelling	0	0	5	4
mesodermal metaplasia	0	0	5	4
epithelial cell nests	0	0	2	2
epithelial pearls	0	0	0	1

(n) = number of membranes examined

TABLE 3

FREQUENCY OF STRUCTURES AND MODIFICATIONS TO THE CHORIOALLANTOIS IN THE PRESENCE OF STAGE 5 EXTRA-EMBRYONIC ENDODERM (AREA OPACA)

	Incubation Period	
Structure	4 days (23)	8 days (22)
keratinization	23	22
ectodermal hyperplasia	23	22
mesodermal swelling	23	22
mesodermal metaplasia	23	22
epithelial cell nests	17	15
epithelial pearls	3	4
yolk sac cords	1	3
cysts	6	4

(n) = number of membranes examined

TABLE 4

FREQUENCY OF STRUCTURES AND MODIFICATIONS OF THE CHORIOALLANTOIS IN THE PRESENCE OF STAGE 22 EXTRA-EMBRYONIC ENDODERM (AREA VITELLINA)

	Incubation Period	
Structure	4 days (11)	8 days (14)
keratinization	11	14
ectodermal hyperplasia	11	14
mesodermal swelling	11	14
mesodermal metaplasia	11	14
epithelial cell nests	6	9
epithelial pearls	1	2
yolk sac cords	1	2
cysts	4	8

(n) = number of membranes examined

spheres were frequently observed within the graft. Peripheral donor cells were large and hypertrophied. The cytoplasm stained lightly when treated with hematoxylin and eosin, or Mallory-Heidenhain preparations (see Figure 10). Matrix material was present in the central portions of the grafts; it was eosinophilic, and did not stain with PAS, Alcian Blue, or Mallory-Heidenhain stains. Occasionally, peripheral cells of the graft exhibited the characteristic morphology of yolk sac cells (Juurlink and Gibson, 1973).

The erosion of the chorioallantoic membrane in the region below the graft was frequently observed. Cords of cells were found in the mesoderm in the proximity of these eroded areas. The cords were composed of large cells, possessing spongy cytoplasm and large eccentric nuclei. Aggregations of fibroblasts surrounded the cords, forming incomplete capsules (see Figure 11).

Although cell cords were associated with eroded portions of the chorionic epithelium, not all eroded sections had cords beneath them. The non-epithelial organization of the cords, and the weak staining reaction suggested that the donor tissue had formed yolk sac. No evidence was found for the presence of any other endodermal derivatives, such as intestine or gizzard.

Necrotic cysts were noted in the mesodermal region of some host membranes. They were surrounded by large, basophilic cells whose size and staining reactions were very similar to those of the chorionic epithelium. The cysts contained an eosinophilic matrix; yolk spheres were occasionally seen. Neither viable cells, nor cellular elements were seen in any of the cysts. Figure 12 shows examples of cysts in the chorioallantois.

Autoradiography was used to determine whether donor or host tissues formed either the yolk sac cords or the necrotic cysts. Figure 13 shows a typical result. Only necrotic cysts were observed in these experiments. The radioactive label was within the cysts, and was not present in the edge cells. This indicated that the cysts were of donor origin, and that the edge cells were from the host.

TABLE 5

FREQUENCY OF STRUCTURES AND MODIFICATIONS TO THE CHORIOALLANTOIS IN THE PRESENCE OF STAGE 5 EXTRA-EMBRYONIC ENDODERM (AREA OPACA): AUTORADIOGRAPHY

Structure	Unlabelled (3)	Sham-labelled (5)	^{3}H-labelled (4)
keratinization	3	5	4
ectodermal hyperplasia	3	5	4
mesodermal swelling	3	5	4
mesodermal metaplasia	3	5	4
epithelial cell nests	2	2	3
epithelial pearls	0	0	0
yolk sac cords	0	0	0
cysts	0	3	3

(n) = number of membranes examined

Discussion

Control hosts and those hosts that received experimental tissues have many features in common. Such features include keratinization, hyperplasia of the chorionic epithelium, mesodermal swelling, mesodermal metaplasia, and the presence of both epithelial cell nests and epithial pearls. Previous investigations - Danchakoff, 1920; DeLanney and Ebert, 1962; Ganote *et al.*, 1964; Goldsworthy and Moppett, 1935; Huxley and Murray, 1924; Moscona, 1959a, 1959b, 1960; Wilt and Stolz, 1962 - have shown that these changes are attributable to inoculation trauma and fenestration of the eggshell.

Keratinization of the ectodermal epithelia of host chorioallantoic membranes is associated with an increase in the relative oxygen concentration within the egg. Moscona (1959a, 1959b, 1960) provided conclusive evidence supporting this hypothesis by incubating fenestrated host embryos in a vapor-saturated atmosphere containing 100 per cent, ninety-five per cent, or ninety-two per cent oxygen. Pure oxygen increased both the degree and the rate of keratinization. Ninety-two per cent oxygen eliminated the keratogenic response completely. Thus, incubation of fenestrated host eggshells in a vapor saturated atmosphere, containing a maximal concentration of ninety-two per cent oxygen, would eliminate the problem of keratinization. In addition, larger fenestra could be cut into the host shells, permitting less traumatic inoculation procedures to be used.

The present inoculation procedure appears to generate chorionic hyperplasia, mesodermal swelling, and mesodermal metaplasia. Wilt and Stolz (1962) indicated that both tissue homogenates and saline

promoted hyperplasia and '... a wide variety of metaplastic responses.' They suggested these reactions were caused by tissue debris, and were therefore a result of '... physical rather than specific chemical stimuli.' Van Haeften (1958) described 'inflammatory' responses to inoculation, and suggested that they were pathological and indicative of a degeneration process. The results of the present investigation support the argument that the reactions are the result of inoculation trauma.

Epithelial cell nests and epithelial pearls have been reported in essentially all previous investigations involving chorioallantoic grafting. Van Haeften believes cell nests to be '... downgrowths of rows of epithelium cells, leading to the formation of chains of cell nests ...' The studies of DeLanney and Ebert (1962) support this conclusion. The force of inoculation fluid striking the chorioallantois is probably responsible for the downgrowth. Absence of these structures in non-inoculated eggs may be taken as evidence supporting this view.

Epithelial pearls are present at the interface of the mesodermal and ectodermal components of the chorioallantois, and at the interface of the graft and the chorionic epithelium. Since these are the only two regions accessible to the atmosphere within the eggshell, we can assume that epithelial pearls are probably keratinized epithelial cell nests and that keratinization is due to their brief exposure to the oxygen-rich atmosphere.

Control tissues from the nodal region of stage 5 embryos differentiated within the mesoderm of the host chorioallantoic membranes.

Extra-embryonic tissues failed to penetrate the chorionic epithelium. Necrotic cysts and yolk sac cords were the only exceptions to this general observation.

The association of yolk sac cords and erosion of the host chorioallantois suggests that the extra-embryonic cells lyse the host membrane. Yolk sac cells are highly phagocytic, and would be expected to contain high levels of intracellular lysozymes (Bellairs, 1963). The release of these enzymes onto the host chorioallantois may be a result of cell death, or of cell differentiation on the surface of the chorioallantois. Interaction between the donor extra-embryonic endoderm and the host's extra-embryonic mesoderm thus requires prior lysis or damage of the host's chorionic epithelium.

Autoradiography showed that the necrotic cysts were of donor origin. The presence of unlabelled host cells at the periphery of the cysts suggests that the donor cells had entered the chorioallantois by encapsulation. Figure 14 shows one possible method of encapsulation, based on models of wound healing (Majbach and Rovee, 1972).

The mechanism by which the control tissues enter the chorioallantois is unknown. It is clear, however, that the cells from the region of Hensen's node do not lyse the host's chorionic epithelium, nor do they become encapsulated. It is reasonable to conclude that there is at least one additional mechanism by which cells may enter the mesodermal region of the chorioallantois.

Individual host membranes exhibit either yolk sac cords or necrotic cysts. No host membranes were found that contained both cords and cysts. This suggests that there is a physical difference

between the grafts. The most obvious difference between grafts is size. The range in size for area opaca sections was from 6 to 12 while area vitellina ranged from 4 to 9 mm^2. Although measurements of individual grafts were not recorded, it seems that the smaller grafts are most likely to be encapsulated, while the larger grafts might be expected to remain on the surface of the chorioallantois. Maibach and Rovee (1972) indicated that wound healing processes are dependent in part on the size of the wound. It is probable that the ability of chorioallantoic grafts to penetrate the chorioallantois is also dependent on the size of the graft. Future experiments might explore the relationship between graft size, and the ability of the graft to enter the membrane.

While the ability of extra-embryonic endoderm to penetrate the chorioallantoic membrane appears to be related to the size of the graft, the mechanisms used are clearly not the same as those of the control tissues. This suggests that extra-embryonic endoderm is in fact different from the control tissue, and that the differences are not due to graft size alone.

Control and experimental tissues differ in two respects: the pattern of vascularization induced by their presence on the chorioallantois, and their ability to penetrate the chorioallantois. Vascularization of control nodal region tissues consisted of several looped vessels which penetrated the graft directly. Conversely, grafts of extra-embryonic endoderm were vascularized by one or two main vessels, and several small vessels that did not penetrate the graft.

All control tissues penetrated the chorioallantois, and differentiated, forming neural elements and notochord. Extra-embryonic endoderm failed to penetrate the chorioallantois. A review of the literature suggested that the ability of a tissue to penetrate the chorioallantois and differentiate in the mesoderm may be related to the mitotic rate and the 'mitotic potential' (ability to undergo mitosis) of the cell population.

Previous investigators have used chorioallantoic grafting to study cellular differentiation. All the tissues used in these studies shared one property - they are all capable of rapid cell division. This is not unexpected, since differentiation is usually associated with cell division (Lash, 1974) especially during early organogenesis. The inability of yolk sac endoderm to penetrate the chorioallantois may indicate that these cells are not capable of cell division at all.

Bennett (1973) indicated that there was a decrease in the mitotic index of cells in the extra-embryonic endoderm. Her data showed that the peripheral nuclei were ninety-five per cent labelled with tritiated thymidine, while more proximal cells had labelling indices of thirty per cent, decreasing to four per cent in the region of the yolk stalk. Unfortunately, Bennett did not provide any estimate of the length of the cell cycle. Altman and Katz (1976) provided data on cell cycle transit times, and mitotic indices for several mammalian tissues. Fully differentiated adult tissues, embryonic tissues and most tumors have mitotic indices of approximately four per cent.

The crucial difference between differentiated cells and embryonic or malignant cells is not the mitotic index, but the transit time. Erythroblastic cells, embryonic cells and malignant cells all have transit times between seven and thirty hours. Epithelial cells from the skin or cornea, on the other hand, have transit times of over one hundred hours. If transit time is related to the ability of a tissue mass to penetrate the chorionic epithelium, it is clear that the transit time for the yolk sac endoderm must be greater than the critical period required for cell penetration.

The lack of studies on early yolk sac differentiation is distressing. The work of Bellairs (1963) and the statements of Ruth (1978) that the region is highly mitotic, or 'blastema-like' suggests a high mitotic rate. Unfortunately, the rate of these divisions is unknown. It is possible that the bulk of the yolk sac endoderm is in fact relatively quiescent, undergoing only two or three mitotic divisions. Newly formed yolk sac may be fully differentiated, histologically and functionally, within hours of its formation.

Evidence that mitotic rate and mitotic potential may be involved in the ability of a tissue to penetrate the chorioallantois is provided by Scher *et al.* (1976). Using adult fibroblasts (NIH/3T3), Scher *et al.* studied that ability of virally-transformed cells, untransformed cells, virally infected cells and normal cells to penetrate the chorioallantois. Transformed cells penetrated the chorioallantois within six hours; non-transformed, virally infected cells and uninfected cells did not penetrate the membrane. Since the transformed cells

have a higher mitotic rate (smaller transit time) than the adult fibroblasts that were not transformed, the work of Scher *et al.* supports the hypothesis that mitotic rate and mitotic potential are involved in the ability of cells to penetrate the chorioallantois.

The patterns of vascularization suggest that the yolk sac endoderm is a fully differentiated tissue. Folkman (1975) noted that fully differentiated tissues, including skeletal muscle, hyaline cartilage and myocardium did not have the capacity to induce angiogenesis when grafted to the chorioallantois. Conversely, embryonic tissues, virally-transformed cells, and several metastatic tumors stimulated angiogenesis. Moreover, differentiated tissues were vascularized by vessels that did not loop; embryonic and malignant tissues were vascularized by looping vessels. It is not possible at present to correlate either mitotic rate or mitotic potential with the ability of a tissue to induce angiogenesis. It is probable, however, that all these phenomena are related to cell surface properties exhibited by the cellular populations.

In conclusion, the failure of extra-embryonic endoderm to penetrate the chorioallantois, and the pattern of vascularization induced by this tissue strongly suggest that extra-embryonic endoderm lacks the ability to undergo rapid cell division. In fact, the tissue is probably fully differentiated, both histologically and functionally, as early as stage 5. Thus, extra-embryonic endoderm is not capable of forming embryonic endodermal derivatives, at least under the conditions imposed by chorioallantois grafting.

CHAPTER TWO

SEROLOGICAL STUDIES OF EXTRA-EMBRYONIC ENDODERM

Introduction

Studies of the antigenic composition of the hypoblast of unincubated stage XIII chicken embryos (Eyal-Giladi and Kochav, 1976) revealed the presence of antigens that were specific for hypoblast alone. Wolk and Eyal-Giladi (1977) indicated that specific antisera were useful in tracing morphogenesis of avian embryos. Schechtman (1947) and Nace and Schechtman (1948) used specific antisera to study the antigens of developing chick embryos. Ebert (1950) suggested that bicchemical variations during ontogeny might be followed, or even blocked by the use of specific antisera. Additional evidence that immunological techniques might be readily adapted to the use of the developmental biologist was provided by Ebert (1951) and Mun (1958).

The major advantages of the use of immunological techniques, compared to histochemistry and biochemistry are the high degree of specificity of the reaction between an antigen and its antibody, and the sensitivity of the tests. Immunological techniques permit the detection of specific antigens at concentrations approaching one microgram, whereas histochemical and biochemical techniques are usually restricted to concentrations in the range of one milligram. Two of the most commonly used immunological techniques are immunodiffusion and immunofluorescence.

Immunodiffusion is based on the classical precipitin reaction (Roitt, 1974). In this reaction, an antigen solution is gradually added to a standard volume of antibody, and a precipitate is formed. This precipitation is maximal at the equivalence point, so named

because it is the point where the concentrations of the antibody and antigen are optimal for producing precipitation. The equivalence point for any one set of antigens and antibodies varies with their source, and the pH and temperature of the reaction mixture (Roitt, 1974). For agar gel immunodiffusion, solutions of antigens and antibodies are placed in wells cut into the gel, and allowed to diffuse towards each other. A precipitate, clearly visible in the gel as a line, forms in the region of the equivalence point. The pattern of the precipitation bands in the gel can be analysed to determine the relationships between several antigens.

Immunofluorescence was developed by Coons (cited in Nairn, 1964). Fluorescent dyes, usually fluorescein or rhodamine, are linked to antibodies, using various chemical techniques. Application of the fluorescence labelled antibodies to tissue sections or cell smears allows the antibodies to bind to specific antigens in the tissue preparations. Antigens can be localized by observing the location of bound fluorescent antibodies with ultraviolet light.

In this section of the thesis, an attempt is made to detect and localize extra-embryonic endodermal antigens, using both agar gel immunodiffusion and immunofluorescence.

Materials and Methods

Antisera against stage 5 area opaca and stage 22 area vitellina were raised in mature female San Juan rabbits after the method of Eyal-Giladi (1977). Initial immunizing preparations consisted of ten stage 5 a. opacae or four stage 22 a. vitellinae homogenized in a saline-adjuvant solution (1 mL Pannett and Compton saline + 0.5 mL Freund's Complete Adjuvent (Calbiochem)). Subcutaneous injections were administered at two week intervals. A series of intravenous injections was initiated during the seventh week. Rabbits received injections or immunogens homogenized in 1 mL Pannett and Compton saline, as indicated in Figure 15. Test bleedings were performed during the ninth week; final bleedings were carried out at the end of the sixteenth week. Blood samples were collected in heparin (Heparin sodium, Fischer Scientific Co.) and centrifuged at 250 x g to remove red blood cells.

Gamma globulin fractions were prepared as per Cempbell *et al.* (1970). One mL aliquots of saturated ammonium sulphate were adjusted to pH 7.8 with 1 N NaOH and added to 2 mL serum samples. The mixture was stirred for thirty minutes at room temperature, then centrifuged at 250 x g in an International clinical centrifuge. The precipitate was dissolved in borate-saline (see Appendix I) (pH 8.5), bringing the sample volume to 2 mL. Precipitations were repeated three times. Following the third centrifugation, the sample was resuspended in 1 mL of borate-saline.

Gamma globulin fractions were dialysed against large volumes of borate saline to remove contaminating sulphate ions. Dialysate was changed every twelve hours, and checked for the presence of sulphate

using barium chloride. The formation of insoluble barium sulphate as a precipitate indicated incomplete dialysis. Dialysis was continued for twenty-four hours after the first negative test for sulphate. The dialysed gamma globulin fractions were centrifuged briefly, and concentrated to a final volume of 5 mL with polyethylene glycol (Carbowax, m.w. = 200,000, Fischer Scientific). Gamma globulin fractions were stored at -10°C.

Precipitating antigen-antibody systems were detected by the immunodiffusion technique of Ouchterlony (Campbell, 1970). Twenty microlitre aliquots of specific antisera were tested against twenty microlitre aliquots of antigen preparations. Agar plates were developed for seventy-two hours at 5°C, and examined for the presence of precipitin bands. Antisera were not diluted; antigen solutions are described below.

Yolk and egg white protein solutions were prepared from fresh, fertile eggs. Chalaza-free egg white was collected in a beaker, frozen, lyophilized, and stored at room temperature as a crystalline powder. Egg yolks were collected in culture dishes and the embryos were removed from their surfaces. The yolks were broken, and lipids were extracted in a solution of acetone and chloroform (1:1, v/v). The upper, organic layer was discarded and the flocculant precipitate retained. Precipitates were air-dried, lyophilized, and stored at room temperature. Two milligrams of yolk or egg white protein were mixed with 1 mL Pannett and Compton saline for use in agar plates.

Homogenates of a. opacae and a. vitellinae were prepared in the manner previously described for the initial immunogen preparations.

Embryonic chick serum was obtained from the vitelline vein of eleven day old embryos. Serum was obtained by centrifuging the collected blood at 250 x g for 10 minutes. The supernatant was retained and used as serum; the cellular pellet was discarded. Peripheral blood was collected from the wing vein of a laying hen and allowed to clot. The serum was collected, and cleared by brief centrifugation. Both adult and embryonic serum were stored frozen.

Specificities of the antisera were determined by absorption with antigen solutions. Two milligrams of yolk or egg white protein were mixed with 1 mL of specific antiserum. The mixture was stirred at room temperature for one hour, then placed in the cold (5°C) for twenty-four hours. Precipitate was removed by centrifugation and the absorption was repeated. Embryonic and adult chicken sera were mixed in a 1:1 (v/v) ratio with the specific antisera. Homogenates of stage 5 a. opacae and a. pellucidae, and stage 22 a. vitellinae were prepared in 1 mL of Pannett and Compton saline and mixed 1:1 (v/v) with the specific antisera. Absorptions were carried out in the manner described for the yolk and egg white proteins. An antiserum was considered absorbed if no reaction was observed when the antiserum and the absorbing antigen were tested in the agar plates.

Immunofluorescence

Several fractions of gamma globulin were conjugated to fluorescein isothiocyanate as described in Nairn (1964). One volume of serum was mixed with two volumes of cold bicarbonate buffer (pH 9.0, 0.5 M) (see Appendix I). Powdered fluorescein isothiocyanate (Sigma) was added to this solution (3 mg/mL buffer) with continous stirring.

Mixing was continued for twelve hours at 5°C. Free fluorescein was removed from the reaction misture by passing the solution through a Sephadex G-15 column (Pharmacia), previously equilibrated with phosphate bufferred saline (pH 7.1, 0.01 M; see Appendix I). Labelled gamma globulin was collected in the void volume, while free fluorescein was retained on the column. Labelled gamma globulin was stored frozen.

Direct immunofluorescence was used to demonstrate the presence of cellular antigens. Labelled gamma globulin was reacted directly with the cell preparations. Antigenic sites recognized by the labelled antibody are localized by the presence of the fluorescent antibody. In this technique, sections of tissues were prepared as recommended by Sainte-Marie (1962). Whole a. opacae and sections of a. vitellinae were fixed in chilled 95% ethanol for twenty-four hours, transferred to chilled 100% ethanol for four hours, then placed in chilled xylene for three hours. During the last hour, the xylene was permitted to come to room temperature. Tissues were imbedded in filtered paraffin at 56°C and stored in the cold. Sections were cut at 5 microns, floated briefly on a water bath, mounted on slides and dried for one hour on a warming tray. The slides were stored in a Bakelite box at 56°C.

Freeze substitution was also used to observe cellular antigens. Fresh tissues were placed on small foil strips and plunged into viscous isopentane (BDH) for thirty seconds. The tissues were removed and immediately placed in n-butanol, prechilled to -42°C (Wigglesworth, 1978). After 48 hours, the tissues were transferred

to fresh n-butanol (also prechilled to -42°C) and allowed to warm slowly to room temperature. A period of four hours was required for the rewarming process.

The tissues were then transferred to a 1:1 (v/v) mixture of n-butanol and ester wax (BDH 1960) (Steedman, 1947; Hancox, 1957). After two hours at 48°C, the tissues were transferred to fresh ester wax and left under vacuum for twenty-four hours. Blocks were poured, allowed to cool slowly at room temperature, and stored at 5°C. Sections were cut at 8 microns, floated on 85% ethanol and allowed to dry at room temperature for twenty-four hours.

Freeze-substituted and paraffin embedded sections were deparaffinized in chilled xylene, rinsed in 100% ethanol, and placed into 100 mL cold phosphate buffered saline (pH 7.0, 0.05 M) (see Appendix I) containing 2 mL of a specific antiserum. Slides were stained for ninety minutes in the buffer-antiserum solution, then rinsed three times in cold buffer. Following mounting in glycerol, slides were stored horizontally in clean Bakelite slide boxes at 4 to 6°C.

Smears of embryonic and adult chicken blood were prepared, along with smears of adult bone marrow, adult liver cells, embryonic a. opacae, a. vitellinae and a. pellucidae. The embryonic tissues were dissociated in cold Pannett and Compton saline on ice (Phillips, 1978). Embryonic blood was obtained from the sinus terminalis of day eleven chick embryos. Adult bone marrow, adult blood cells and adult liver cells were obtained from a laying hen. Bone marrow was collected by forcing a stream of medium L-15 through the marrow cavity of the

femur. Fat was removed by centrifugation at 200 x g; bone marrow cells were resuspended in fresh medium L-15. Adult blood cells were collected by cardiac puncture. Liver cells were obtained by forcing pieces of liver through a fine steel mesh; the cells were collected in medium L-15.

All cell preparations were fixed in formalin, placed on slides and allowed to air dry. Slides were stained in an antiserum-buffer solution as described above. Staining reactions were continued for two hours. Following mounting in glycerol, slides were stored at 4 to 6°C. The length of the staining period had no effect on either the intensity or the degree of staining observed.

Fluorescence microscopy was performed using a Leitz ultraviolet microscope fitted with the following filters: BG 38 (red suppression), UG 5 (UV fluor filter), KG1 (heat shield) and BG 12 (blue suppression). The barrier filter in the ocular tube was a K 530 filter (green suppression).

Control slides were treated with preimmunization serum (both conjugated and unconjugated), fluorescein isothiocyanate solution (3 mg/mL staining buffer) and fluorescein conjugated goat anti-rabbit gamma globulin.

Results

Antisera raised in female San Juan rabbits against extra-embryonic endoderm of stage 5 and stage 22 chicken embryos were examined for specificity using agar gel diffusion. The antigen-antibody systems detected by this method are summarized in Tables 6 and 7. It is apparent that the extra-embryonic tissues share several antigens with adult and embryonic chicken serum, yolk, and egg white proteins. Absorbtion of the antisera with various antigen solutions was used to render the antisera specific for extra-embryonic endoderm.

Antiserum to stage 5 extra-embryonic endoderm recognized at least one antigen common to both yolk and egg white protein, two antigens in adult and embryonic chicken serum, and one antigen expressed by area opaca and area vitellina. Absorbtion of the antiserum with either yolk or egg white protein eliminated one of the precipitin bands (see Table 6). Only one precipitin band, shared by area opaca, area vitellina, adult chicken serum and embryonic chicken serum, remained following absorbtion. The pattern of precipitation suggested that the antigen was common to these four antigen solutions (see Figure 6). Absorbtion with area pellucida had no effect on the precipitin reactions observed, while absorbtion with adult or embryonic chicken serum, or homogenates of extra-embryonic endoderm eliminated the remaining precipitin band.

Antiserum to stage 22 area vitellina was similar to the stage 5 antiserum in that it also recognized antigens in adult and embryonic chicken serum, yolk and egg white protein. Absorbtion with yolk eliminated several antibodies from the antiserum. Two antigens were recognized in the adult serum and area opaca solutions, while only one

in the homogenates may have been a source of the variation. The data reported in Tables 6 and 7 are the maximum numbers of bands observed in three replicate plates.

Absorbed sera were less variable in precipitin tests than unabsorbed sera. The presence of a single precipitin band common to extra-embryonic endoderm, embryonic chicken serum, and adult serum was consistently observed in reactions with antiserum to stage 5 area opaca. Similarly, the precipitin reaction between stage 22 antiserum and adult serum consistently produced two bands, while the reaction between stage 22 antiserum and either extra-embryonic cells or embryonic chicken serum produced only one band.

Immunofluorescence was used to observe extra-embryonic cellular antigens. Freeze-substituted and paraffin sections of extra-embryonic tissues did not exhibit fluorescence under the experimental conditions used. Freshly prepared, formalin-fixed cell smears of area opaca, area vitellina, adult granulocytes and adult bone marrow all reacted positively (see Table 8). Erythrocytes from adult peripheral blood, embryonic erythrocytes (day 11 incubation), adult liver cells and area pellucida cell smears did not exhibit fluorescence (see Figures 17 and 18). There is no obvious difference in the staining reaction of cell smears with either of the two gamma globulin preparations.

Granulocytic cells from adult chicken peripheral blood and bone marrow - mainly heterophils (Lucas and Jamroz, 1961) - stained with the conjugated gamma globulin. The staining reaction was limited to the granules themselves and the perinuclear region of the cytoplasm. Lymphocytes were separated from the blood sample using LSM solution (Bionetics); the lymphoid cells did not react.

antigen was recognized in the embryonic serum and the area vitellina homogenate. Further absorption with egg white protein removed one precipitin band from the reaction between the stage 22 antiserum and area opaca, but had no effect on the other reactions. Absorption with area pellucida did not affect these results, while absorption with embryonic serum, adult serum, area opaca or area vitellina eliminated all the remaining precipitin bands (see Tables 7 and Figure 16).

Control, pre-immunization sera were examined for the presence of specific antibodies, and were absorbed with the antigen solutions in the manner described for the experimental antisera. Precipitin bands were not observed in any control experiment.

These results indicate the presence of several saline-soluble antigens that are shared by the extra-embryonic endoderm adult chicken serum, embryonic chicken serum, yolk and egg white protein. Furthermore, antiserum raised against stage 5 area opaca recognizes at least one antigen that is present only in extra-embryonic endoderm and chicken serum. The antiserum raised against stage 22 area vitellina recognizes two antigens in adult chicken serum, and one antigen in embryonic chicken serum and extra-embryonic cell homogenates.

The agar gel immunodiffusion tests were difficult to replicate. Repeated freezing and thawing of the antisera led to a steady decrease in antiserum potency and a reduction in the intensity of the precipitin bands in the agar plates. The most variable results occurred in the reaction of unabsorbed serum and antigen solutions. This was probably due in part to the fact that fresh antigen solutions were prepared for each replicate plate. Variability between embryos

TABLE 6

SALINE-SOLUBLE ANTIGENS DETECTED BY ANTISERUM TO STAGE 5 EXTRA-EMBRYONIC ENDODERM

	Antigen tested							
Absorbing agent	y	a	y+a	cs	es	ao	av	ap
none	1	1	1	2	2	1	1	0
yolk protein	0	0	0	1	1	1	1	0
egg white protein	0	0	0	1	1	1	1	0
yolk + egg white protein	0	0	0	1	1	1	1	0
adult chicken serum	0	0	0	0	0	0	0	0
day 11 chicken embryo serum	0	0	0	0	0	0	0	0
area pellucida homogenate	0	0	0	1	1	1	1	0
area opaca homogenate	0	0	0	0	0	0	0	0
area vitellina homogenate	0	0	0	0	0	0	0	0

Numerals indicate the number of precipitin bands present in agar plates. y = yolk protein, a = egg white protein, y+a = yolk and egg white protein, cs = adult chicken serum, es = day 11 chicken embryo serum, ao = stage 5 area opaca homogenate, av = stage 22 area vitellina homogenate ap = stage 5 area pellucida homogenate.

TABLE 7

SALINE-SOLUBLE ANTIGENS DETECTED BY ANTISERUM TO STAGE 22 EXTRA-EMBRYONIC ENDODERM

	Antigen tested							
Absorbing agent	y	a	y+a	cs	es	ao	av	ap
none	1	1	1	5	2	2	1	0
yolk protein	0	0	0	2	1	2	1	0
egg white protein	0	0	0	2	1	1	1	0
yolk + egg white protein	0	0	0	2	1	1	1	0
adult chicken serum	0	0	0	0	0	0	0	0
day 11 embryo serum	0	0	0	0	0	0	0	0
area pellucida homogenate	0	0	0	2	1	1	1	0
area opaca homogenate	0	0	0	0	0	0	0	0
area vitellina homogenate	0	0	0	0	0	0	0	0

Numerals indicate the number of precipitin bands present in agar plates. y = yolk protein, a = egg white protein, y+a = yolk and egg white protein, cs = adult chicken serum, es = day 11 embryo serum, ao = stage 5 area opaca homogenate, av = stage 22 area vitellina homogenate, ap = stage 5 area pellucida homogenate.

TABLE 8

SUMMARY OF IMMUNOFLUORESCENCE EXPERIMENTS: CELL SMEARS

γ- globulin Fraction	ae	aly	ale	eb	ap	ao	av	abm	ali
Anti-stage 5 area opaca	-	-	+	-	-	+	+	+	-
Anti-stage 22 area vitellina	-	-	+	-	-	+	+	+	-

- + no reaction; + = positive reaction. ae = adult erythrocytes; aly = adult lymphocytes; ale = adult leucocytes; eb = embryonic blood (day 11); ap = area pellucida; ao = stage 5 area opaca; av = stage 22 area vitellina; abm = adult bone marrow; ali = adult liver.

Both of the extra-embryonic cell types stained lightly in the cytoplasmic region, with intense staining of the yolk spheres. The majority of the fluorescence was clearly associated with the yolk spheres. Fluorescence reactions for all cell types were eliminated by absorbing the labelled gamma globulin fractions with area opaca, area vitellina, adult chicken serum, embryonic chicken serum, adult bone marrow or adult leucocytes. Absorbtion with area pellucida had no effect on the fluorescent reaction. Control sera and solutions did not produce fluorescent staining with any cell preparations.

Discussion

The detection of antigens common to the extra-embryonic endoderm, yolk, and egg white proteins is not surprising, since yolk sac cells have been shown to phagocytose both albumin and yolk proteins (Bellairs, 1963; Romanoff, 1960). Agar immunodiffusion of specific antisera to unincubated germ layers of stage XIII chicken embryos (Eyal-Giladi and Kochav, 1976) showed that the epiblast and hypoblast differ antigenically only in their microsomal constituents. The majority of the detectable antigens were common to the germ (blastoderm) and the extra-embryonic yolk.

Schechtman (1947) reported the presence of '... one or more substances antigenically similar to constituents of the adult serum which are present in the early chick.' Using whole adult chicken serum, and fractions of the adult serum as immunogens, Schechtman produced several antisera. The antisera were tested against chick embryos at stages 5, 8, 9, and 12 (Hamburger and Hamilton, 1951). Precipitation reactions were observed for all embryonic stages tested. Absorbtion of the antisera with yolk protein eliminated the precipitin reactions. Schechtman termed the antigens detected by his antisera 'vitelloid', since they were apparently shared by the yolk. A second set of antigens that were not shared with yolk were also detected. These antigens were termed 'non-vitelloid', and were found to react only with the blood of embryos incubated for six days or longer (Schechtman, 1947).

Nace and Schechtman (1948) studied the appearance of the non-vitelloid antigens in the serum of embryonic chickens, and

found that they appeared in the serum and on embryonic blood cells during the fifth day of incubation. They concluded '... that while substances with vitelloid combining groups are present throughout the life history, others with non-vitelloid combining groups differentiate on about the fifth day of embryonic development.'

The results of the present study indicate the presence of non-vitelloid antigens in embryos as early as stage 5. These antigens are restricted to the extra-embryonic region, as indicated by the failure of the area pellucida to react with the antisera in immunodiffusion tests. The discrepancy between the results of Nace and Schechtman (1948) and the present study may be explained by the differences in the yolk antigen used for absorbtion of the antisera. Schechtman used whole yolk to absorb his antisera. In the experiments reported here, the yolk was treated with an acetone/chloroform solution to remove lipid material. Schjeide *et al.* (1963) noted the presence of several lipoproteins in the yolk of the fowl, and listed oleic acid and triglycerides as major constituents of the macromolecules. Since oleic acid and triglycerides are soluble in the acetone/chloroform mixture, it is possible that a potential absorbing antigen was removed from the yolk solution. The present absorbtion is thus less complete than the absorbtion of Nace and Schechtman.

Antisera raised against stage 5 area opaca detected at least one antigen common to the extra-embryonic cell population and both embryonic and adult chicken serum. Kram and Klein (1976) showed that stage 5 chicken blastodiscs actively synthesize serum proteins.

Serum transferrin, serum albumin, and an alpha globulin were the most common products. Nace, 1951 (cited in Kram and Klein, ibid) stated '... that products present in the yolk served directly as serum proteins.' The immunodiffusion results support this hypothesis.

The detection of a second serum antigen with antisera raised against stage 22 area vitellina may indicate the synthesis of an additional serum protein by the yolk sac endoderm. If this is the case, a reaction between the area vitellina homogenate and the stage 22 antisera could be predicted, although such a reaction was not observed. Low levels of antigen in the endodermal cell homogenates may be the reason for the null reaction.

This hypothesis is supported by Kram and Klein (1976). Measuring the pattern of protein synthesis in yolk sac, they found that at least twenty-five yolk sacs were required to permit detection of nascent proteins. In the present study, four a. vitellinae were used for the antigen preparation. It is possible that the concentration of antigen in the stage 22 area vitellina homogenates was below the equivalence point concentration required for the formation of the precipitin band.

Attempts to observe cellular antigens with immunofluorescence were successful only when freshly-prepared, formalin-fixed cell smears were used. Freeze-substituted and paraffin-embedded material did not exhibit fluorescence. Both these techniques required the use of organic solvents, specifically xylene and ethanol. In addition, heat was required for the embedding of tissues; the minimum heat used was 45°C. Antigen loss through solubilization or denaturation by heat are the most probably reasons for the absence of a fluorescence reaction.

Formalin-fixed smears of stage 5 and stage 22 extra-embryonic endoderm showed strong cytoplasmic staining associated with the yolk spheres. Lysed cells exhibited much stronger fluorescence reactions than non-lysed cells, and this staining reaction remained after yolk absorption. The fluorescent antigen is therefore not a component of the yolk protein solution used for absorption. It is probable that the antigen detected is a lipoprotein or a newly synthesized protein product of the yolk sac cells.

Circulating peripheral granulocytes and bone marrow cells showed an intense fluorescence reaction in the presence of labelled antisera. The pattern of fluorescence observed in the granulocytes indicates that the antigens detected by the antisera are cytoplasmic. Yolk absorption had no effect of this fluorescence reaction; absorption with embryonic or adult serum, extra-embryonic cell homogenates or granulocytes negated the reaction. Thus, it can be concluded that the antigens detected by immunofluorescence are related in some way.

The probable relationship between the antigens detected in embryonic and adult chicken serum has been outlined previously. The relationship between the extra-embryonic cells, serum and granulocytes remains unknown.

In an effort to identify possible developmental relationships, cells from adult liver and erythrocytes from adult chickens and eleven day old chicken embryos were examined for immunofluorescence. The failure of liver cells to fluoresce suggested that the antigen was not present in the adult liver, and that the antigen was not

'endodermal'. Absence of fluorescence by red blood cells from either day eleven embryos or adult chickens indicated that the antigen was not related to the erythrocyte membrane antigens described by Blanchet (1976). Lymphocytes also failed to fluoresce in the presence of specific antisera. The antigen detected in yolk sac endoderm and adult granulocytes is clearly not restricted to the derivatives of one embryonic germ line.

The results of the immunodiffusion and immunofluorescence experiments suggest that more than one antigen had been detected. Immunofluorescence experiments indicated that the antigens were associated with yolk spheres in yolk sac endoderm, and with cytoplasmic granules in the granulocytes. Both cell types are phagocytic, and possess large amounts of lytic enzymes in lysosomes. The antigen may therefore by a lytic enzyme, or a lipoprotein component of a lysosome or a yolk sphere.

Evidence that the antigen may be a lipoprotein is provided by the failure of freeze-substituted and paraffin-embedded tissues to fluoresce. Treatment of these materials with organic solvents would remove lipoproteins, and thus eliminate the fluorescence reaction. Furthermore, lipoproteins were excluded from the yolk antigen mixture used to absorb the antisera. Schjeide *et al.* (1963) lists several lipoprotein components of serum, and notes that two of these components, serum lipovitellin and β-lipoproteins were elevated in the laying hen. Together, the literature and the results reported here suggest that at least one of the antigens detected by the antisera to extra-embryonic endoderm may be a lipoprotein.

The second antigen, detected in adult chicken serum by antiserum to stage 22 extra-embryonic endoderm may be a 'developmental antigen'. It is absent in embryos at stage 5, and may be inferred to be present in the embryos at stage 22. Since the antigen is tissue-specific, and appears to be acquired after a set time period has elapsed, it is reasonable to suggest that the antigen is a marker of extra-embryonic endoderm differentiation. The nature of the antigen remains unknown.

In conclusion, immunodiffusion analysis of antisera directed against yolk sac endoderm indicated the presence of at least two antigens. The first antigen, common to extra-embryonic cells and embryonic and adult chicken serum, is probably a lipoprotein. A second antigen, present in adult chicken serum and probably synthesized by stage 22 area vitellina, represents a developmental antigen. The nature of the second antigen is unknown. Immuno-fluorescence experiments indicated that the antisera bind to antigens associated with cytoplasmic granules in granulocytes, and the yolk spheres of yolk sac endodermal cells. The similarities in functions of the two cells, and in the composition of their cytoplasmic constituents suggests that the antigen is either associated with the membranes of the yolk spheres and granulocyte granules or is a lytic enzyme.

The fluorescence of cytoplasmic granules in the presence of specific antisera should be re-examined. Longenecker (personal communication) noted that cytoplasmic granules frequently bind antisera non-specifically, producing a false positive result. Experiments testing the reactions between cytoplasmic granules and fluorescent

antisera raised against other proteins not normally found in the chick should be performed to confirm the specificity of the fluorescent reactions.

Figure 1. Diagrammatic representation of extra-embryonic membranes of the avian embryo (from Balinsky, 1975).

c = chorion

am = amnion

al = allantois

e = embryo

ys = yolk sac

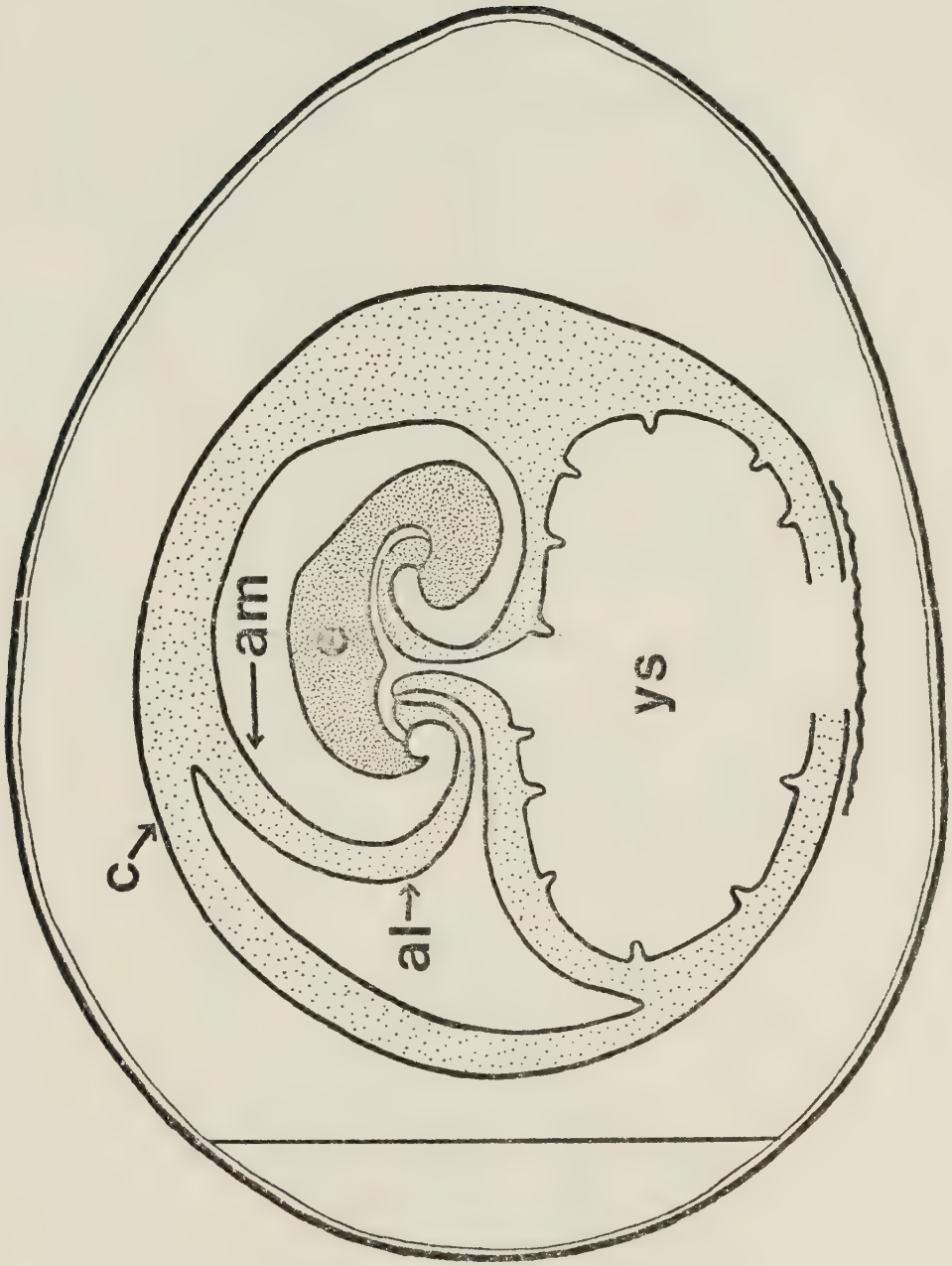

c
am
al
ys

Figure 2. Diagrammatic representation of the chick yolk sac during the third day of incubation.

en = extra-embryonic endoderm

ec = extra-embryonic ectoderm

y = yolk

v = yolk sac villus

m = extra-embryonic splanchnic mesoderm

b = blood island

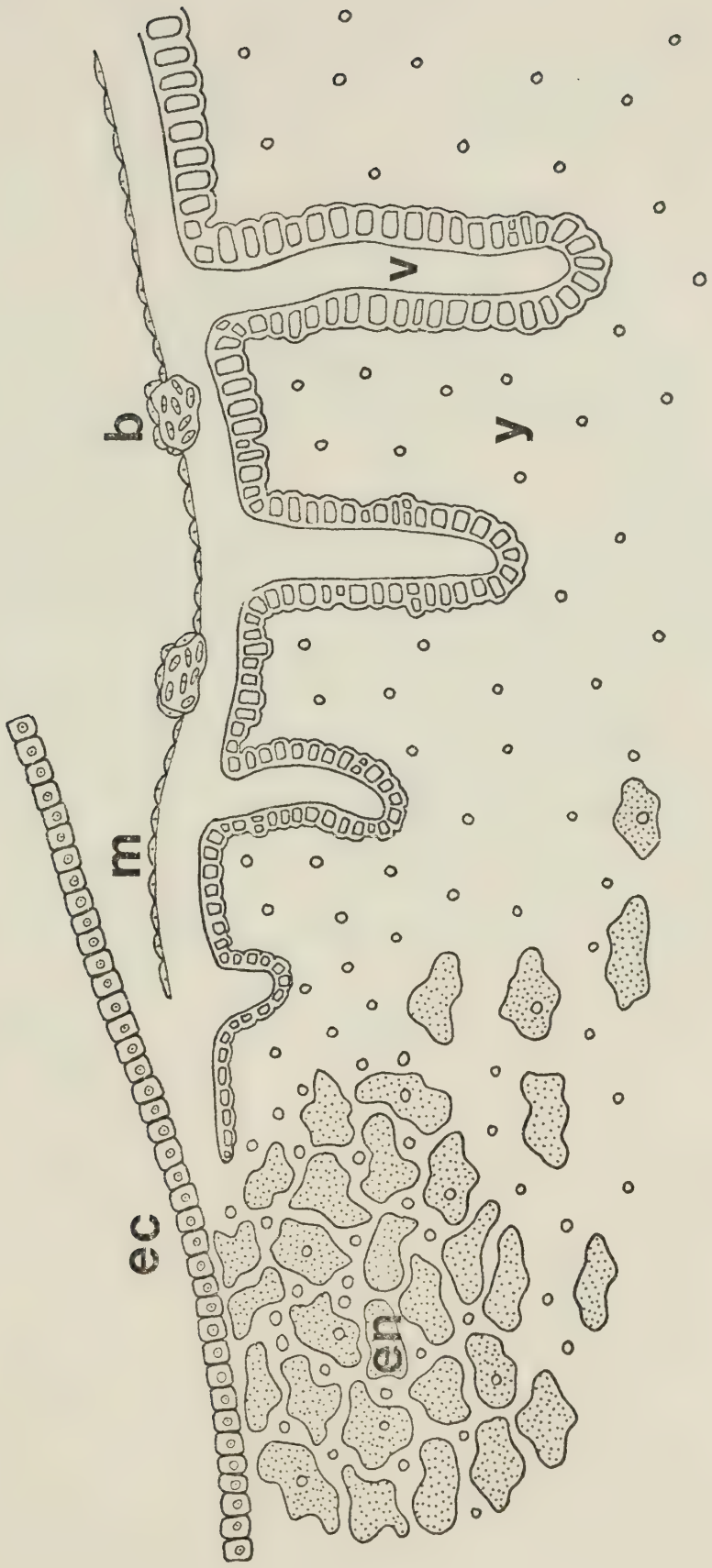

v
b
y
m
ec
en

Figure 3. Diagrammatic representation of the ventral view of a stage 5 embryo. (from Wakely and England, 1978)

en = endoderm

ec = ectoderm

ps = primitive streak

me = mesodermal boundary

Grafts were taken from region directly anterior to horizontal line

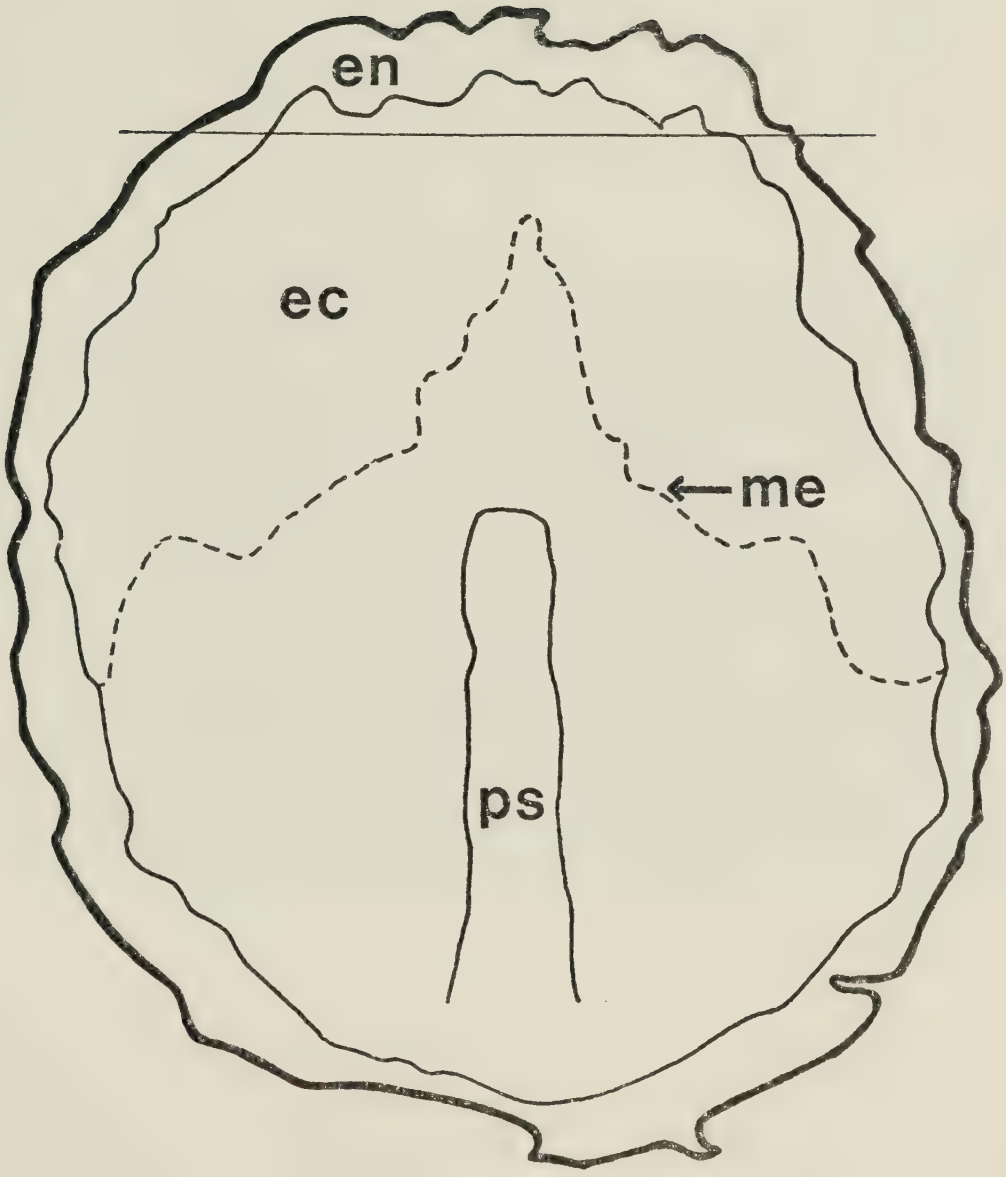

en
ec
me
ps

Figure 4. Graft on chorioallantois (X 7).

g = graft

c = chorioallantois

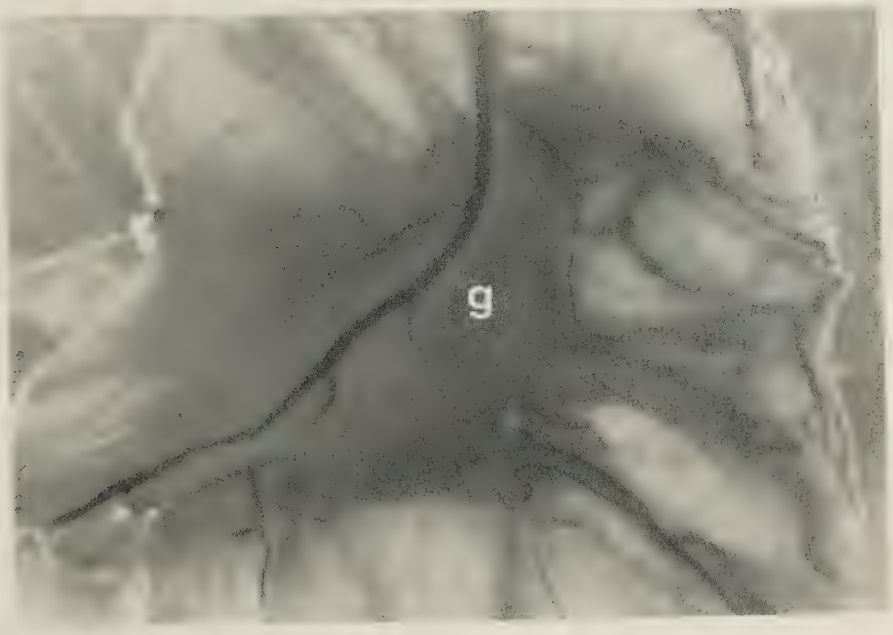

g

Figure 5. Cross-section of control chorioallantois. (X 120)

vs = vascular sinus

ec = ectoderm

en = endoderm

m = mesoderm

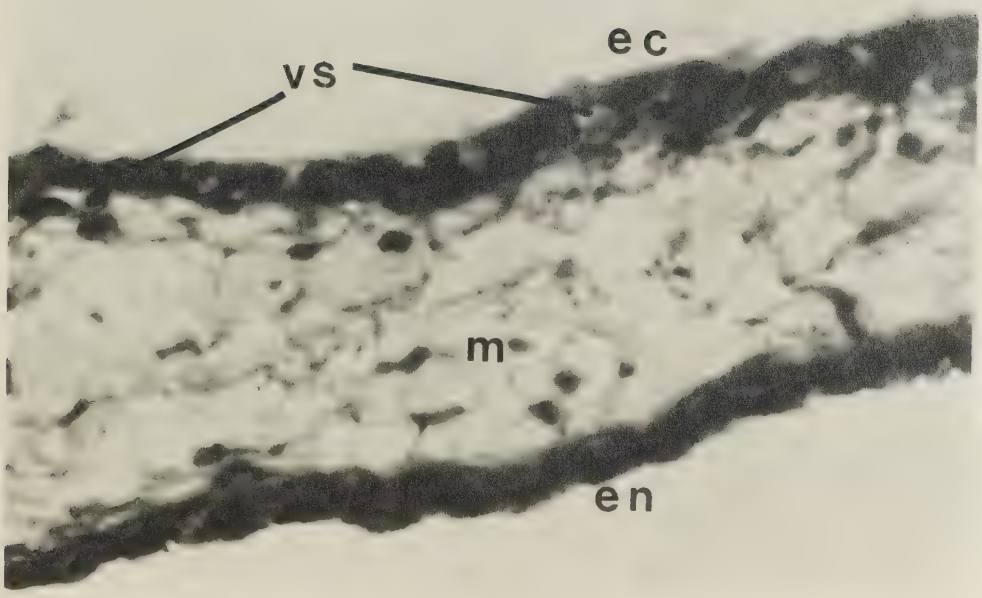

ec
vs
m
en

Figure 6. Papilliform processes formed by host ectoderm. (X 160)

p = papilliform processes

g = graft

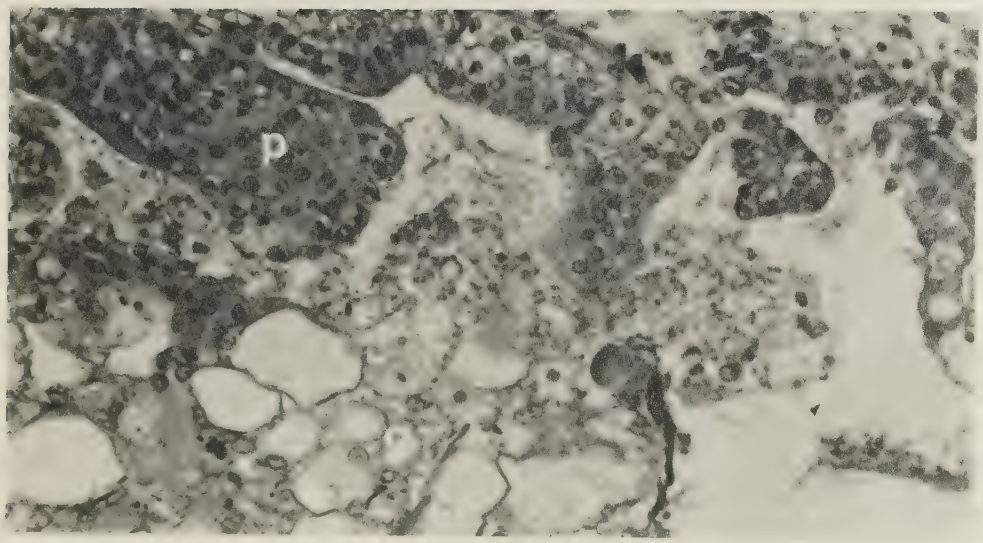

p

Figure 7. Metaplasia of the host chorioallantois. (X 120)

m = bundles of condensed host mesenchyme

s = stromal response: appearance of many fibres in the mesodermal region of the membrane

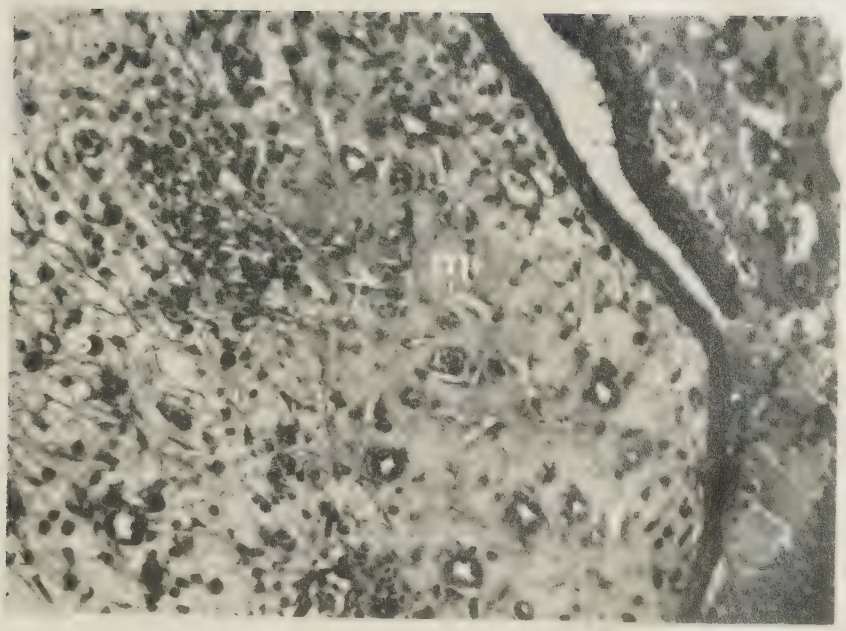


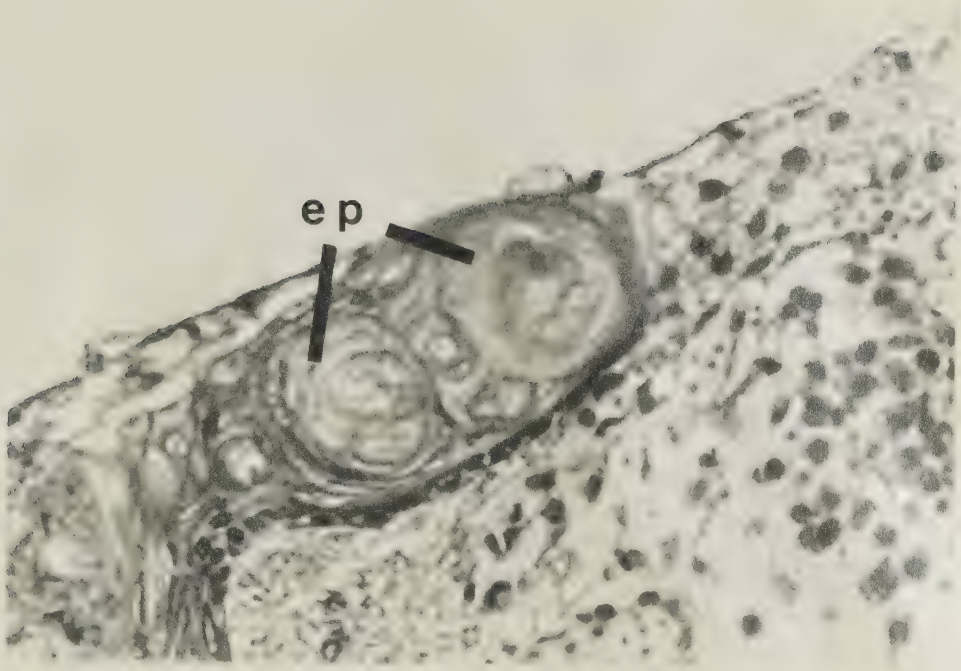
Figure 8. Epithelial pearls and epithelial cell nests.

Upper photo (X 180):

ep = epithelial pearls

Lower photo (X 180):

cn = cell nests


ep

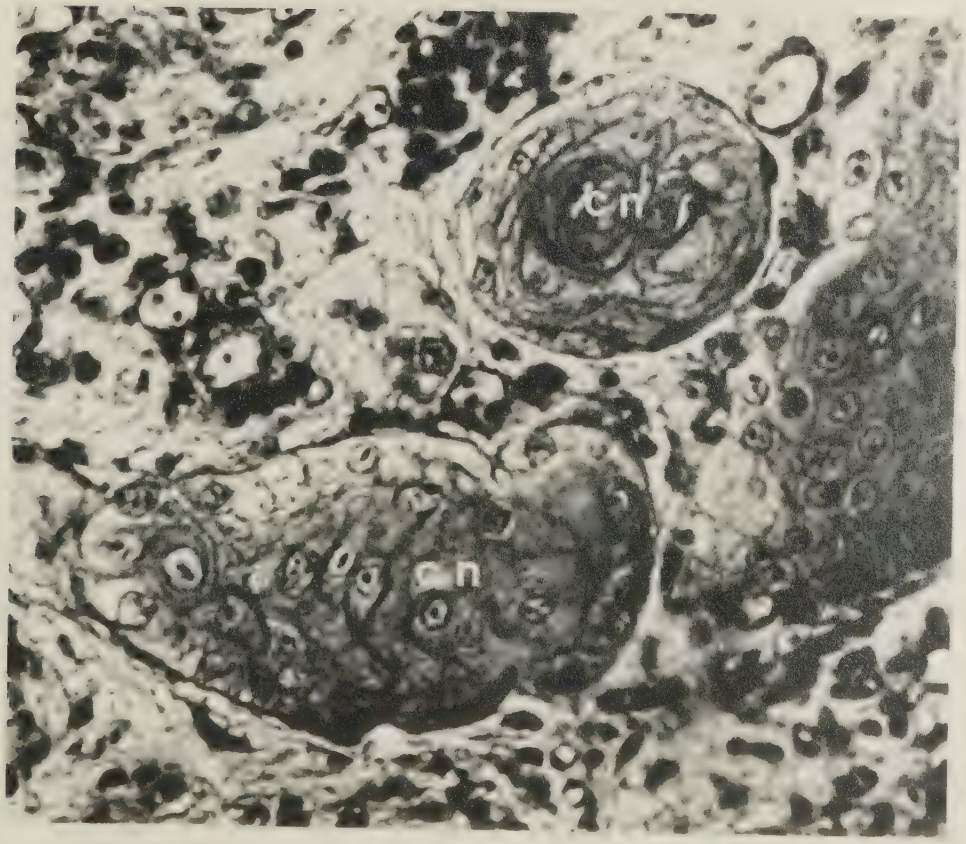

tcn
cn

Figure 9. Control tissues from stage 5 area pellucida within the host chorioallantois.

Upper photo (X 120):

nt = neural tube

nc = notochord

Lower photo (X 480):

Detail of neural tube


nc
nt

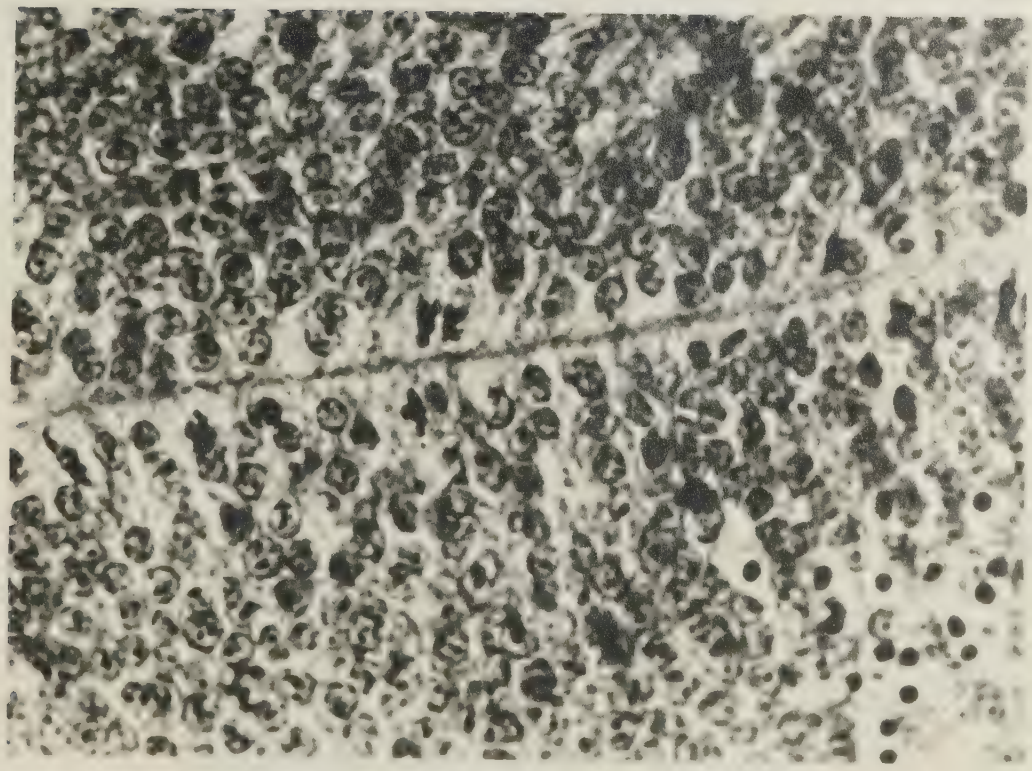


Figure 10. Extra-embryonic endoderm on the surface of the host chorioallantois

Upper photo (X 120):

g = graft

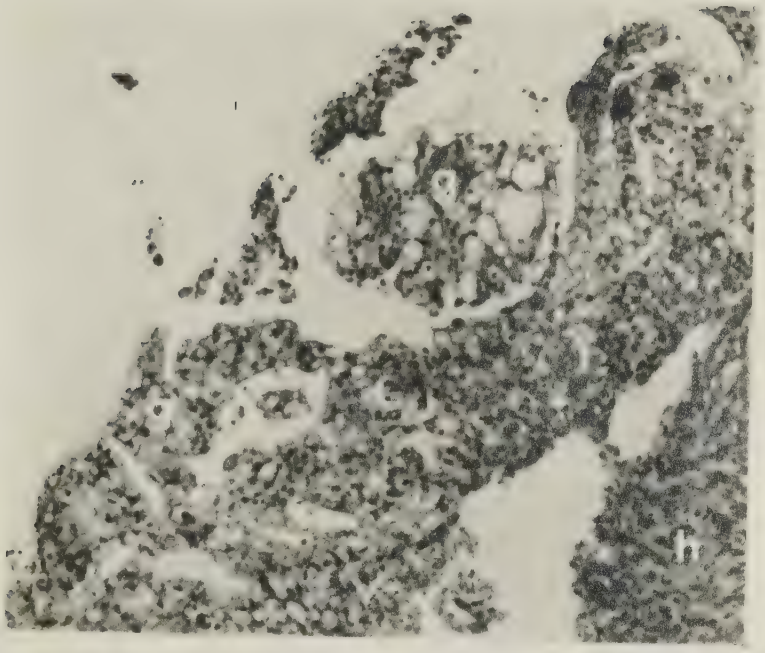
h = host

Lower photo (X 160):

n = necrosis

h = host

e = extra-embryonic endoderm


h

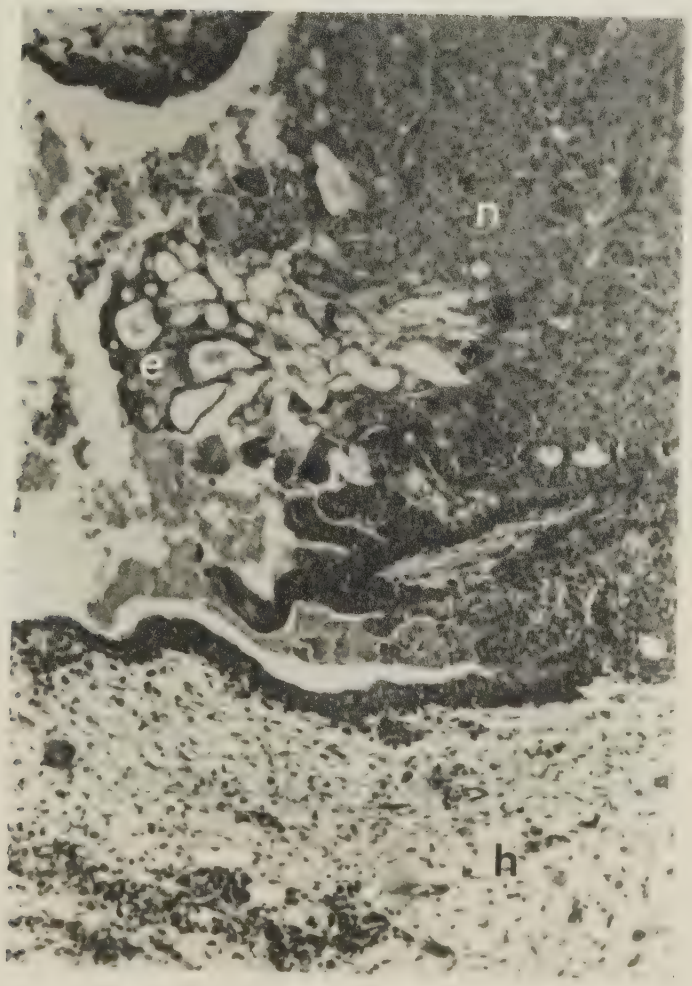
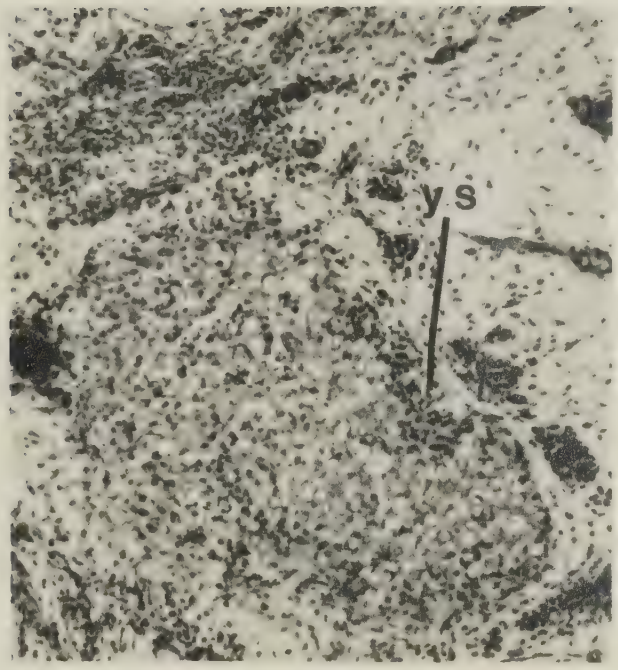

D
e
h

Figure 11. Cell cords within the host chorioallantois (X 120)

ys = yolk sac


y s

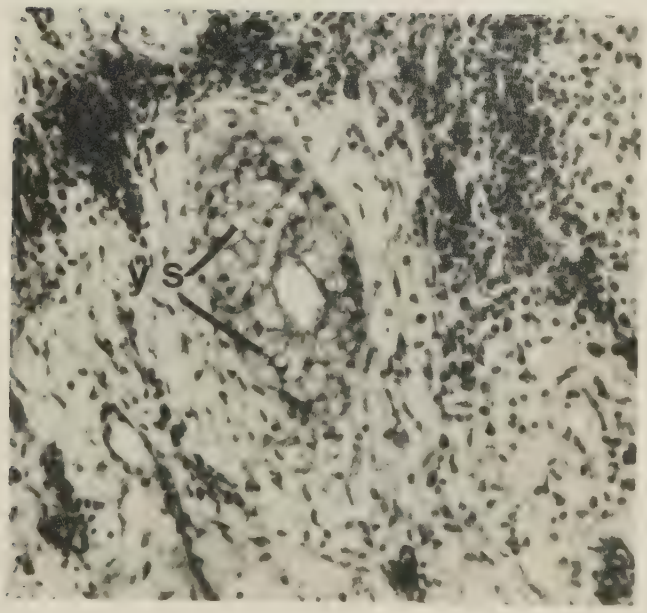
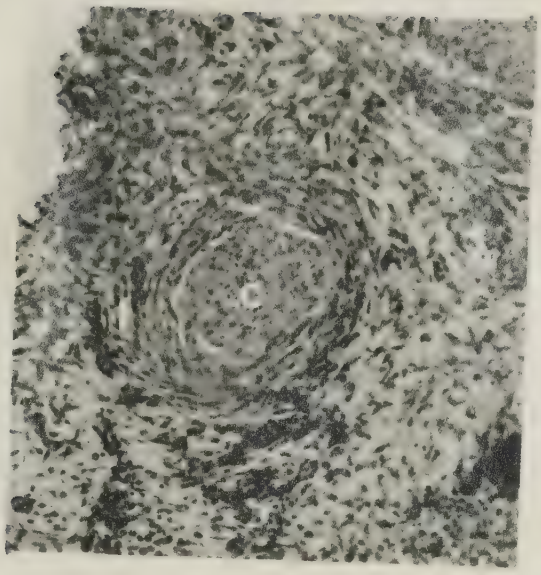

y s

Figure 12. Necrotic cysts within the host chorioallantois (X 120)

c = cyst

f = fibrous capsule



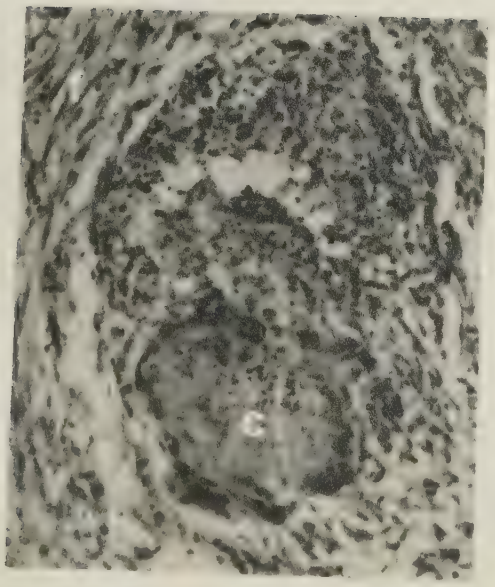


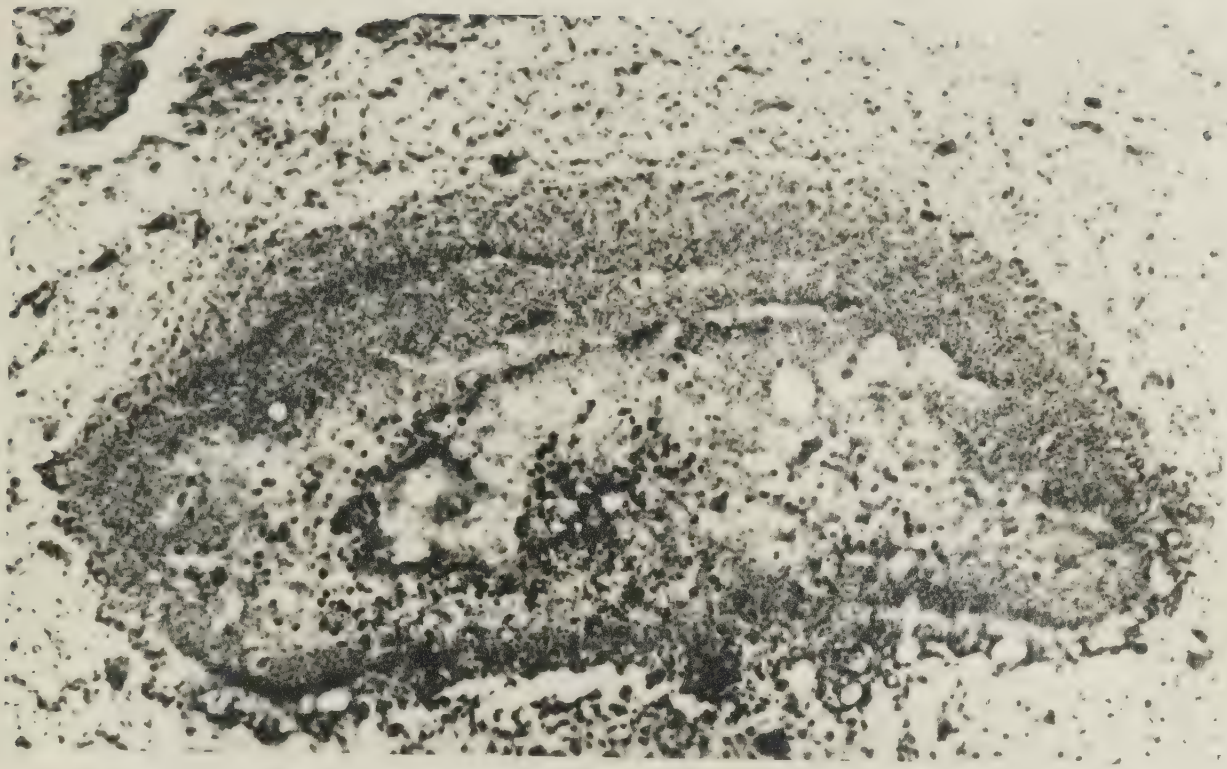
Figure 13. Autoradiography of donor tissue within the host chorioallantois

Upper photo (X 140):

Radioactive label within the necrotic cyst

Lower photo (X 560):

Detail of radioactive label within the necrotic cyst



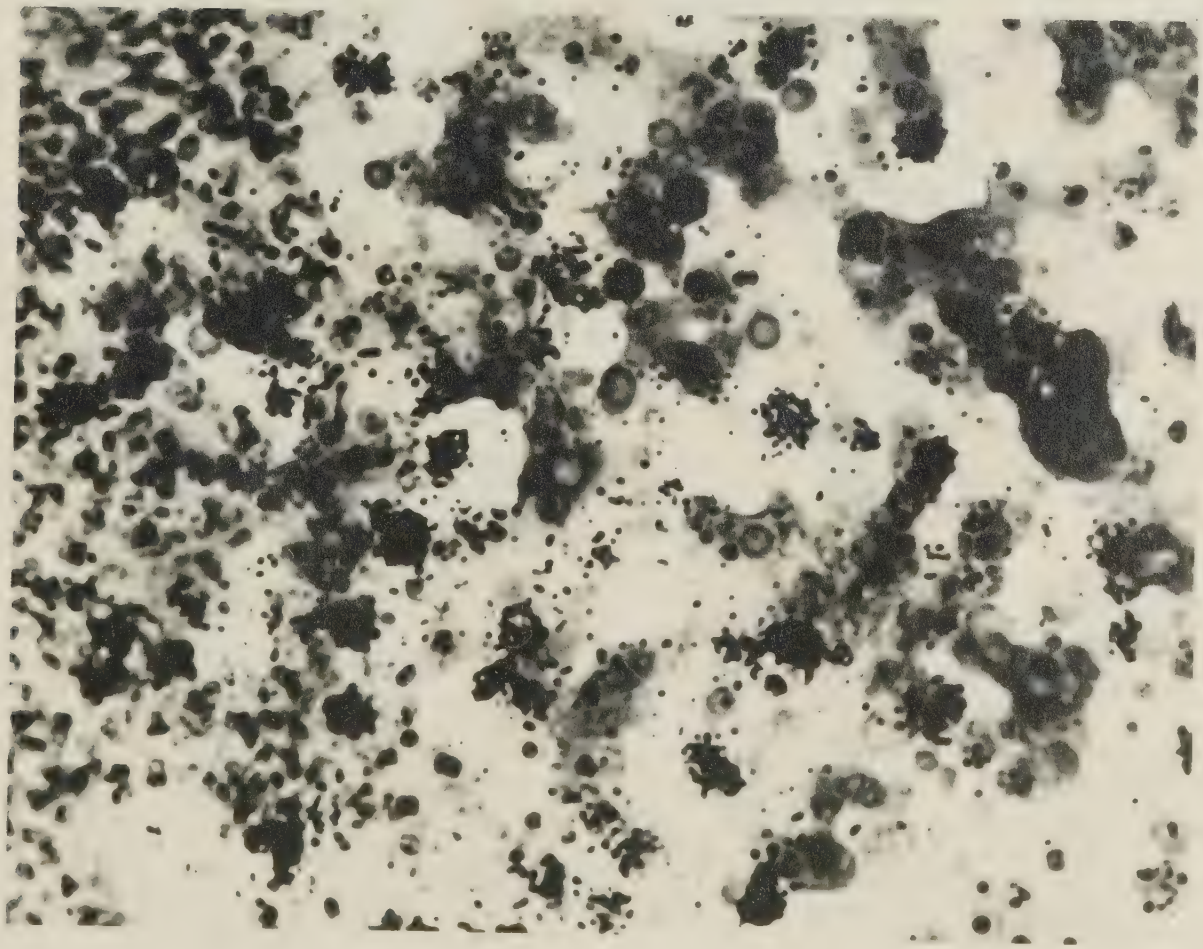


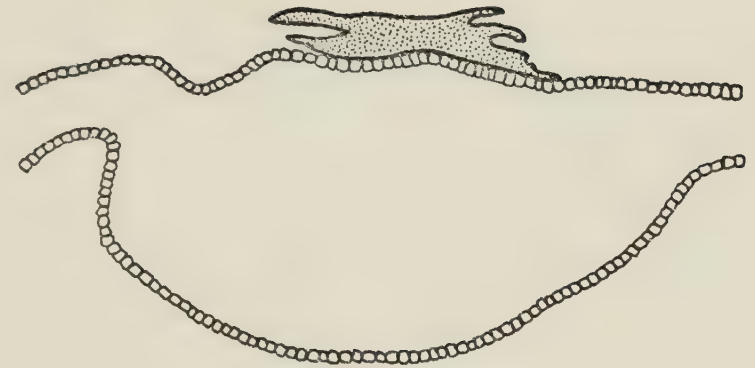
Figure 14. Diagrammatic representation of the encapsulation of donor tissue by the chorioallantois

A. Graft of the surface of the chorioallantois

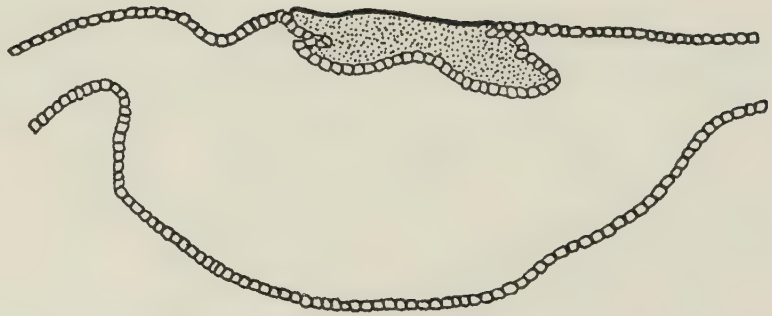
B. Contraction of the chorioallantois in the region below the graft

C. Overgrowth of ectoderm

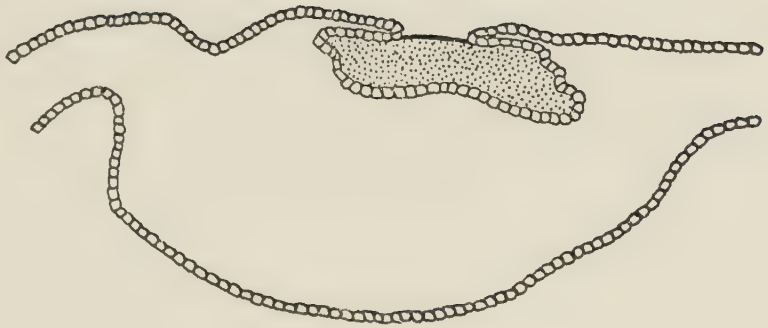
D. Encapsulated graft within the mesoderm



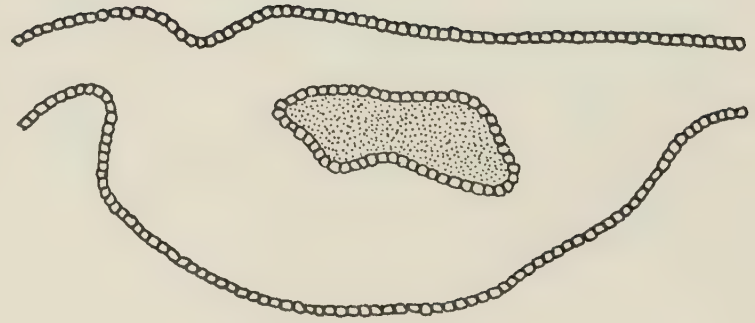
A



B



C



D

Figure 15. Immunization protocol for production of antisera to extra-embryonic endoderm.

IMMUNIZATION PROTOCOLS

	First Stage Immunization Procedure*			Second Stage Immunization Procedure**						
	Week 1	Week 3	Week 5	Week 6	Weeks 7 and 15				Weeks 8 and 16	
					Day 1	Day 3	Day 5	Day 7		
Rabbit #1	10 a. opacae	10 a. opacae	10 a. opacae		3 a. opacae	6 a. opacae	12 a. opacae	24 a. opacae	Rest	Bleed
Rabbit #2	4 a. vitellinae	4 a. vitellinae	4 a. vitellinae		1 a. vitellinae	2 a. vitellinae	4 a. vitellinae	8 a. vitellinae	Rest	Bleed
					Repeat					

Repeat second stage immunization procedure during week 15

* All injections in 1 ml. Pannett and Compton saline mixed with 0.5 ml. Freund's Complete Adjuvant (Calbiochem)

**All injections in 1 ml. Pannett and Compton saline only

Figure 16. Diagrammatic representation of the precipitin reactions of absorbed antisera and several antigens

Upper diagram:

Reaction of antiserum against stage 22 extra-embryonic endoderm and selected antigens

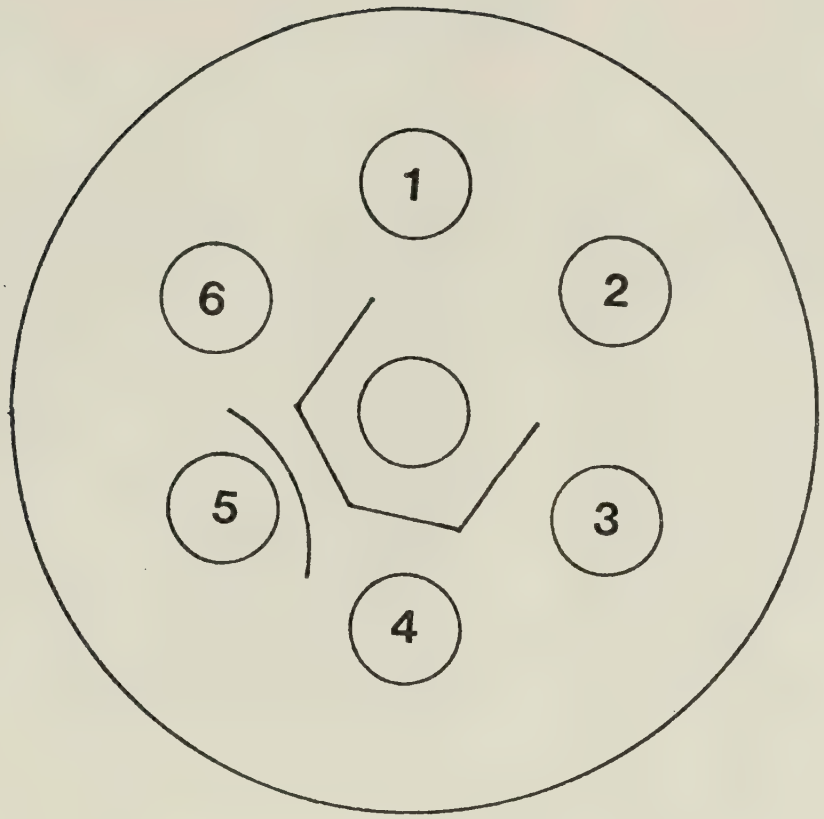
center well = antigens to stage 22 extra-embryonic endoderm

Lower diagram:

Reaction of antiserum against stage 5 area opaca and selected antigens

center well = antigens to stage 5 area opaca

1. yolk and egg white protein
2. area pellucida homogenate
3. area opaca homogenate
4. area vitellina homogenate
5. adult chicken serum
6. embryonic chicken serum


1
2
3
4
5
6

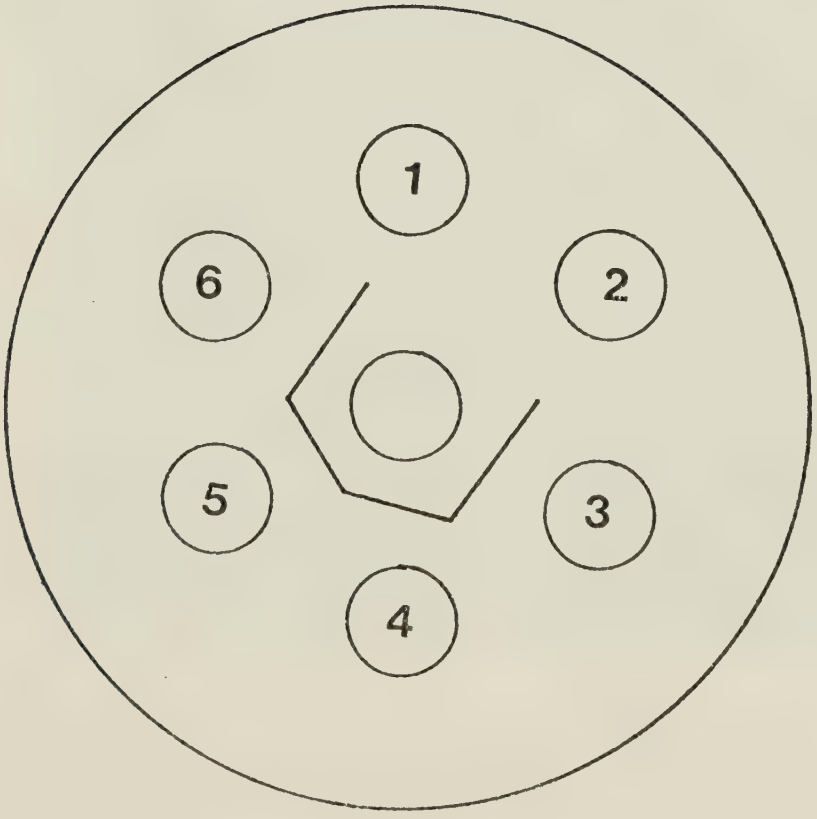

1
2
3
4
5
6

Figure 17. Immunofluorescence of extra-embryonic endodermal tissues

Upper photo, right (X 1100):

Fluorescence of yolk spheres in cytoplasm of lysed extra-embryonic endodermal cell (area opaca)

Upper photo, far right (X 1100):

Extra-embryonic endodermal cell (area opaca)

Lower photo (X 300):

Fluroescence of non-lysed extra-embryonic endoderm (area opaca)

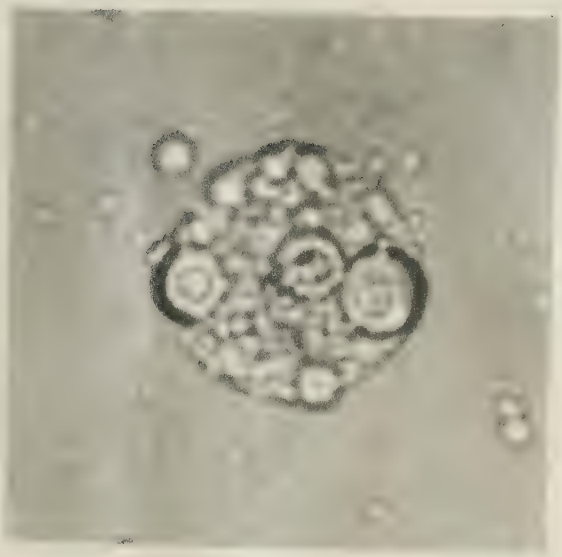


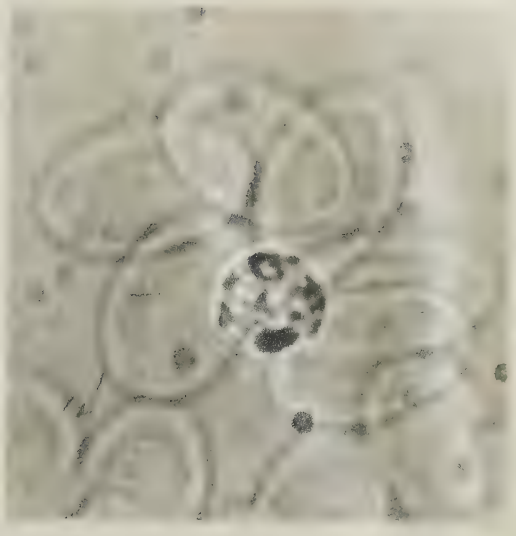
Figure 18. Immunofluorescence of adult granulocytes

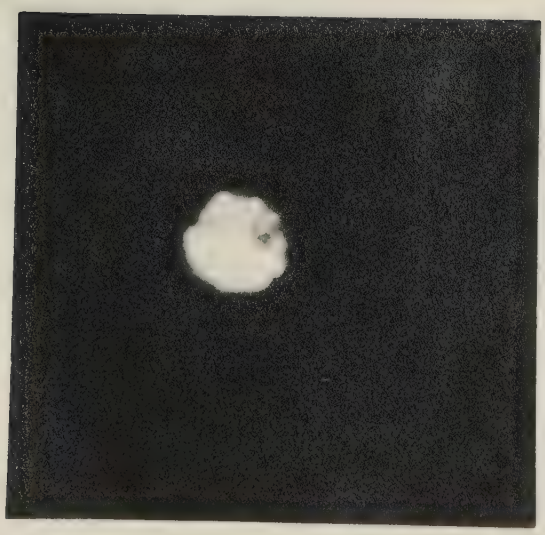
Upper photo (X 650):

Adult granulocyte (tungsten light)

Lower photo (X 650):

Adult granulocyte (ultra-violet light)





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APPENDIX I

COMPOSITION OF SALINE SOLUTIONS AND BUFFERS

All buffers and saline solutions were prepared with de-ionized, triple distilled water.

Pannett and Compton Saline

sodium chloride	82.8 mM
potassium chloride	8.3 mM
calcium chloride	2.7 mM
magnesium chloride, hexahydrate	2.5 mM
N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid	15 mM

Borate Saline

boric acid	100 mM
borax	25 mM
sodium chloride	75 mM

Carbonate-Bicarbonate Buffer

sodium bicarbonate	145 mM
sodium carbonate	5.6 mM

Phosphate-Buffered Saline

sodium chloride	145 mM
disodium hydrogen phosphate	7.5 mM
sodium dihydrogen phosphate	2.5 mM

APPENDIX II

HISTOLOGICAL STAINS

Ehrlich's Hematoxylin and Eosin

Solution 1

hematoxylin	2.0 gm
aluminum alum ($NH_4Al(SO_4)_2.12H_2O$)	3.0 gm
ethanol	100 mL
glycerol	100 mL
sodium iodate	0.24 gm
acetic acid (glacial)	10 mL
distilled water	100 mL

Solution 2

Eosin Y	1.0 gm
ethanol	1000 mL
acetic acid (glacial)	5 mL

Mallory-Heidenhain

- to 200 mL of distilled water, add the following in the order given:

phosphotungstic acid	1.0 gm
Orange G	2.0 gm
aniline blue	1.0 gm
acid fuschin	3.0 gm